CORE
Metadata, citation and similar papers at core.ac.uk
Provided by Repositório Aberto da Universidade do Porto

Joana Catarina Pereira Jorge Fonseca da Rocha

Biomarcadores ecotoxicológicos na espécie exótica invasiva *Corbicula fluminea:* possíveis contribuições da Ecotoxicologia para a Medicina Legal

Dissertação de Candidatura ao grau de Mestre em Medicina Legal submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

Orientador – Professora Doutora Lúcia Guilhermino

Categoria – Professora Catedrática

Afiliação:

- Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto, Departamento de Estudos de Populações, Laboratório de Ecotoxicologia

- Centro Interdisciplinar de Investigação Marinha e Ambiental, Laboratório de Ecotoxicologia e Ecologia.

Agradecimentos

Em primeiro lugar gostaria de agradecer à Professora Doutora Lúcia Guilhermino, por tudo aquilo que me transmitiu durante todos estes anos em que trabalhamos em conjunto, desde o meu estágio de Licenciatura, à bolsa de investigação que tive sob sua orientação, e até agora à minha tese de Mestrado. Agradeço-lhe todas as oportunidades que me deu, assim como por ter acreditado em mim e nas minhas competências, permitindo-me trabalhar a seu lado todos estes anos.
Agradeço também à Professora Maria José Pinto da Costa, por todo o conhecimento, apoio, carinho, compreensão e amizade, que sempre estiveram presentes.

O trabalho experimental conducente à presente Tese de Mestrado foi financiado por Fundos FEDER através do Programa Operacional Fatores de Competitividade – COMPETE e por Fundos Nacionais através da FCT – Fundação para a Ciência e a Tecnologia no âmbito dos projetos "NISTRACKS – Processos que influenciam o comportamento invasor da espécie não indígena *Corbicula fluminea* (MOLLUSCA: BIVALVIA) em estuários – identificação de factores genéticos e ambientais chave" (PTDC/AAC-AMB/102121/2008; FCOMP-01-0124-FEDER-008556) e "PEst-C/MAR/LA0015/2011", e por verbas próprias do Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto, atribuídas ao Departamento de Estudos de Populações e ao Laboratório de Ecotoxicologia em particular. A candidata teve uma bolsa de iniciação à investigação científica (BIC) no âmbito do projeto NISTRACKS.

Resumo

A Medicina Legal, no contexto atual, é uma área multidisciplinar, utilizando conhecimentos e ferramentas de muitas outras ciências incluindoDireito, Psiquiatria, Psicologia, Biologia, Genética, Toxicologia, Ecologia e Ecotoxicologia. No âmbito da Ecotoxicologia e das suas potenciais contribuições para a Medicina Legal, inclui-se a monitorização da poluição, a qual pode ter efeitos adversos, incluindo letais, nos seres vivos e no Homem. Determinar o estado de saúde de populações de espécies selvagens em relação à contaminação ambiental tem interesse preventivo relativamente à saúde humana e pode ser um contributo circunstancial determinante em casos de fatalidades humanas. Assim, com o objectivo de contribuir para o aperfeiçoamento de metodologias para uso em programas de monitorização e outros estudos com interesse para a Medicina Legal, os objectivos destes estudo foram (i) caracterizar as colinesterases (ChE) presentes em diversos tecidos da espécie exótica invasora *Corbicula fluminea* (Mollusca: Bivalvia) das populações das áreas estuarinas de água doce sob influência da maré dos rios Minho e Lima (NW da Peninsula Ibérica), e (ii) validar a metodologia para avaliação do estado de saúde de populações selvagens de *C. fluminea* em áreas estuarinas de água doce sujeitas à influência da maré, utilizando diversos biomarcadores ambientais integrados através de um índice de stresse (IBR). As enzimas presentes no músculo aductor, na hemolinfa e nas brânquias de *C. fluminea* foram inibidas significativamente pelo sulfato de eserina, pelo inibidor específico da acetilcolinesterase (AChE) (BW284C51), no caso do músculo aductor e da hemolinfa, e pelo inibidor específico da pseudocolinesterase (PChE) de mamíferos (iso-OMPA). Estes resultados indicam que as enzimas presentes nestes tecidos não podem ser classificadas como AChE ou PChE, uma vez que possuem propriedades típicas de ambas, pelo que devem ser designadas por ChE. No músculo do pé, a actividade enzimática medida parece ser predominantemente de esterases não específicas, uma vez que em geral não se verificou inibição por sulfato de eserina. O estudo de monitorização, efectuado ao longo de um ano permitiu dosear com sucesso os níveis de peroxidação lipídica indicador de danos oxidativos; e a actividade das seguintes enzimas: esterases no músculo do pé, envolvidas na biotransformação de xenobióticos; isocitrato desidrogenase e octopine desidrogenase envolvidas na produção de energia; glutationa S- transferases envolvidas na biotransformação e na prevenção de stresse oxidativo; catalase, glutationa reductase e glutationa peroxidase que são componentes das defesas anti-oxidantes. Observou-se uma variação sazonal em todos os parâmetros sublinhando a importância de ter este factor em consideração em estudos de biomonitorização utilizando esta espécie como

bioindicador. A integração dos dados dos biomarcadores utilizando o IBR indicou stress relativamente baixo em todas as estações do ano, com um máximo de 5.58 no Verão e um mínimo de 1.8 no Inverno. Assim, em estudos de biomonitorização com esta espécie, devem ser usados tecidos particulares de *C. fluminea* para medir a actividade das ChE ou das esterases, preferencialmente ao corpo total; deve ser tida em consideração a variabilidade sazonal dos biomarcadores estudados, sendo estes adequados para avaliar o estado de saúde de *C. fluminea*, e o local monitorizado no rio Minho pode ser utilizado como local de referência em futuros estudos, uma vez que os espécimens apresentam níveis relativamente baixos de stress ambiental.

Palavras-chave: espécies invasoras não-indígenas, *Corbicula fluminea,* caracterização colinesterases, biomonitorização, stress oxidativo, enzimas relacionadas com energia

Abstract

Nowadays, Forensic Medicine is a multidisciplinary science, which uses knowledge and tools from many other sciences, such as Law, Psychiatry, Psychologu, Biology, Genetics, Toxicology, Ecology and Ecotoxicology. In the scope of Ecotoxicology and its possible contribuitions for Forensic Medicine, one can include the pollution monitoring, which can have adverse and even lethal effects in the Human being. Assessing health status of wild species populations in relation to environmental contamintation, is of interest for preventive measures for human health and can be a circunstamcial contribute of interest in cases of human fatalities. Therefore, and with the objective of contribuiting to the enhancing of metodologies for use in monitoring programmes and other studies of interest to Forensic Medicine, the objectives of this studie were to (i) characterize the cholinesterases (ChE) present in different tissues from the exotic invasive species Corbicula fuminea (Mollusca: Bivalvia) from populations of the freshtidal waters areas of Minho and Lima rivers (NW Iberian Peninsula), and (ii) to validate the methodology used for health status assessment of wild populations of *C. fluminea* in freshtidal waters areas using various environmental biomarkers that were later integrated by a stress index (IBR). The enzymes present in the adductor muscle, haemolymph and gills of C. fluminea were significantly inhibited by eserine sulphate, acetylcholinesterase (AChE) specific inhibitor (BW284C51), for aductor muscle and haemolymph, and pseudocholinesterase (PChE) specific inhibitor in mammals (iso-OMPA). These results show that the enzymes present in this tissues cannot be classified as AChE or PChE, as they show properties typical of both forms, thus should be referred to as ChE. As for foot tissue, the measured enzymatic activity is apparently predominantly from non specific esterases, as inhibition by eserine sulphate could not be verified. The biomonitoring study, assessed through a year long period, allowed the sucessful assessment of lipid peroxidation (indicator of oxidative damage), and the activity of the following enzymes: esterases in foot tissue, envolved in xenobiotics transformation; isocitrate dehydrogenase and octopine dehydrogenase, involved in energy production; gluthatione-S-transferases, involved in biotransformation and oxidative stress prevention; catalase, gluthatione reductase and gluthatione peroxidase, components of anti-oxidative defenses. A seasonal variation was observed for all parameters, highlighting the importance of considering this factor in biomonitoring studies using this species. The integration of data obtained from biomarkers showed a relative low stress in every season, with a maximum of 5.58 in Summer and minimum of 1.8 in Winter. Therefore, in biomonitoring studies with this species, specific tissues from *C. fluminea* should be used to measure ChE or EST activities instead of

using the whole body; seasonal variability should be considered when assessing *C. fluminea* health status, and the monitored site in Minho river can be used as a reference in future studies, as specimens show relative low values of environmental stress.

Keywords: exotic invasive species, *Corbicula fluminea,* cholinesterase characterization, biomonitoring, oxidative stress, energy related enzymes

Index

List of abbreviations

AChE – Acetylcholinesterase
BHT - Butylated hydroxytoluen
BW284C51 - 1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide
CAT - Catalase
CBD – Convention on Biological Diversity
CDNB - 1-chloro-2,4-dinitrobenzene
ChE - Cholinesterases
DNA - deoxyribonucleic acid
DTNB - 5,5'-dithiobis-(2-nitrobenzoic acid)
DTPA - diethylene triamine pentaacetic acid
DTT - Dithiothreitol
EDTA - Ethylenediaminetetraacetic acid
ERA – Environmental Risk Assessment
EST - Esterases
GPx – gluthatione peroxidase
GR – gluthatione reductase
GSH - reduced glutathione
GSSG - oxidized gluthathione
GST – gluthatione-S-transferases
IBR – Integrated Biomarker Response Index
IDH – isocitrate dehydrogenase
iso-OMPA - N,N´-diisopropylphosphorodiamic acid
LDH – lactate dehydrogenase
LOOH - lipid hydroperoxide
LPO – lipid peroxidation
$NADP^+$ - nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH - nicotinamide adenine dinucleotide phosphate (reduced form)
NIS – non native invasive species
ODH – octopine dehydrogenase
PChE - pseudocholinesterase
ROS – reactive oxygen species
TBARS - thiobarbituric acid reactive substances
TFW - tidal freshwater area
USA – United States of America

List of figures

Chapter I – General Introduction

Chapter II – Characterization of the Cholinesterases Present in Specific Tissues of Corbicula fluminea (Mollusca: Bivalvia) From Two Estuaries of the NW Iberian Coast

Chapter III – Biomonitoring and Seasonal Variability of Corbicula fluminea Selected Biomarkers in the Minho River Estuary

List of tables

Chapter I – General Introduction

Chapter II – Characterization of the Cholinesterases Present in Specific Tissues of Corbicula fluminea (Mollusca: Bivalvia) From Two Estuaries of the NW Iberian Coast

Chapter I – General Introduction

1.1. Forensic Medicine and Ecotoxicology

The modern Forensic Medicine is a multidisciplinary science, using knowledge, approaches and tools from several other areas such as law, psychiatry, psychology, biology, genetics, toxicology and ecology (França, 2005). In the last decades, the use of environmental data and evidences to help on the establishment of circumstances leading to human fatalities has been increasing (Carmichael *et al.*, 2001; Markert *et al.*, 2008; Pimentel *et al.*, 1980). In this sense, the "new" science of Ecotoxicology is expected to provide a major contribution for Forensic Medicine in the next future. It can help, for example, in establishing the circumstances of dead through the identification of particular toxins and/or organisms present in the local of the fatality, using animal models in ecotoxicological assays to diagnose modes of toxic action, symptoms and mortality due to the exposure to the agent(s) suspected of having cause the human fatality, among other possibilities. However, a considerable amount of work still needs to be done to take more advantage of the knowledge, evidences and tools that Ecotoxicology may offer to Forensic Medicine.

And what is Ecotoxicology? Ecotoxicology is a science of a broad scope; it studies the adverse effects of chemical substances (environmental contaminants), either natural or synthetic, and other stressors (e.g. temperature) on ecosystems, their biota, and the environment in general (Peakall & Walker*,* 1994). In addition to its one knowledge, approaches, techniques and tools, it uses those from several other sciences, especially from Toxicology, Ecology, and Environmental Chemistry. It investigates adverse effects at different levels of biological organization from the molecular to the ecosystem level (Peakall & Walker*,* 1994). Important tools in Ecotoxicology are laboratory bioassays carried out to investigate the effects of single or mixtures of environmental contaminants in specific organisms or model ecosystems, and monitoring programs in defined ecosystems to assess the effects of stressors over organisms, populations, communities and ecosystems in real scenarios over time.

1.2. Environmental Biomonitoring

Biomonitoring is the repeated use of assessment/observation/evaluation of chemical, physical or biological parameters, that are measured using tested, confirmed, comparable and standardized methods, according to a predetermined schedule over time and space (Walker *et al.,* 2006). Data from chemical, physical or biological measured environmental parameters may be combined and used as an indicative of environmental quality,

monitoring the levels of environmental contaminants or the health status of wild populations (Peakall, 1992).

According to Walker *et al.* (2006), biomonitoring can be divided into four main approaches: (i) community effects; (ii) bioconcentration of pollutants; (iii) effects of pollutants; and (iv) genetically based resistance to pollution. In aquatic ecosystems, routine measurements in the water column often include various and different abiotic parameters such as temperature, salinity and dissolved oxygen, nutrients, biological measurements of responses (in individuals, populations or communities, for example) and chemical analysis to determine the presence of chemical contaminants. Ideally, programmes integrating biological, physical and chemical analysis should be carried out because they will provide the maximum of information regarding the ecological status.

1.3. Biomarkers

Along the years, various definitions of the term biomarkers have been suggested, but the general and common idea is that these tools are used to try to connect biological effects and the potential hazard that population might be subjected to (Figure 1) (Bucheli & Fent, 1995). Despite that the link between molecular damages and effects at a population level is not a straight relationship (Walker *et al.*, 2006) environmental biomarkers are an attempt to enrich this knowledge. Biomarkers indicative of oxidative stress, biotransformation, physiologic stress and alterations in the pathways of energy production are often of interest (Table 1).

Table 1 - Biomarkers commonly used in biomonitoring studies with aquatic species.

Biomarker	Function
Lipid peroxidation (LPO) levels	indicator of oxidative damage
Esterases (EST) activity	xenobiotic transformation
Catalase (CAT) activity	anti-oxidative defenses
Gluthatione peroxidase (GPx)	
Gluthatione reductase (GR)	
Glutathione-S-transferases (GST) activity	involved in biotransformation and oxidative stress prevention
Octopine dehydrogenase (ODH) activity	energy production
Isocitrate dehydrogenase (IDH) activity	
Acetylcholinesterase (AChE)	assessment of neurotoxicity

And how can one assess pollutant exposure? Various tools are available, but oxidative stress is one of great relevance due to the potential damage caused by reactive oxigen species (ROS): it can range from a neurological level to behavioural changes, including endocrine disruption, genotoxicity, effects on reproduction and others (Vasseur & Cossu-Leguille, 2006).

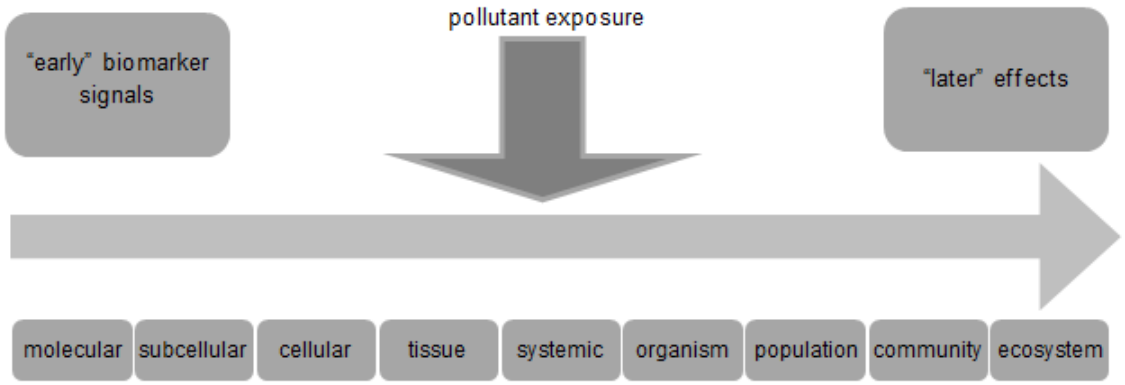

Figure 1 - Scheme of pollutant exposure and the level of effects that can occur (adapted from van der Oost *et al.,* 2003).

An approach to infer on oxidative damage is the measurement of antioxidant system and the level of tissue damage that ROS produce (Walker *et al.*, 2006). This can be quantified by enzymatic levels of certain antioxidant enzymes such as catalase (CAT), gluthatione peroxidase (GPx) and glutathione reductase (GR). Another commonly used enzymes are gluthatione-S-transferases (GST), that facilitates the biotransformation of metabolites by polarizing them and thus making the excretion process easier. Other processes can also be assessed, like lipid peroxidation (LPO), DNA damage, energetic metabolism alterations measured by activity of enzymes like lactate dehydrogenase (LDH) and isocitrate dehydrogenase (IDH). Investigations can also include neurotoxic effects that can be assessed with the activity of cholinesterases (ChE), among other parameters.

CAT catalyses a very important reaction for removal of ROS, it helps the removal of H_2O_2 by metabolizing it to H_2O (water) and O_2 (oxygen), being very specific to H_2O_2 (Stegeman *et al.*, 1992). GPx, a enzyme belonging to the peroxidases family, that also transforms H_2O_2 into water, by the means of a co-factor. This transformation occurs from the oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) and acts as a protection from lipid peroxidation (van der Oost et al., 2003). This is vital for the equilibrium although GPx is not directly involved in the process of detoxification like CAT (Winston & Giulioz, 1991). Glutathione reductase (GR) reduces GSSG to GSH, allowing

disponibility of GSH for the reaction of GPx, and also oxidizes nicotinamide adenine dinucleotide phosphate (NADPH) to its oxidized form ($NADP^+$) (Blokhina *et al.*, 2003).

The organism antioxidant defenses, although efective when dealing with small amounts of ROS, cannot cope with significantly high amounts, leading to peroxidation of polyunsaturated fatty acids, or lipid peroxidation (LPO), which is one of the most important consequences known (Stegeman *et al.*, 1985). This process involves several chain reactions, being of a very extensive nature, but it can be summarized as follows. There is a formation of lipid radicals and consequently, of lipid hydroperoxide (LOOH), resulting in a peroxidized membrane, losing permeability and integrity (Valavanidis *et al.,* 2006). One possible outcome is the adverse pathological conditions that can affect the organisms. LPO is usually measured through the quantification of thiobarbituric acid reactive substances (TBARS).

Toxic damage can have other consequences besides lipid peroxidation, it can affect also the energy production of particular pathways depending on oxygen conditions. Two enzymes related with the Krebs cycle can be utilized for this particular matter: isocitrate dehydrogenase (IDH) and on lactate dehydrogenase (LDH). IDH is one enzyme that can be studied for aerobic paths for the production of energy and also has an important role in the antioxidant system as its reaction produces NADPH, a cofactor for the reaction of GR (Lima *et al*., 2007). As for anaerobic pathways, one can rely on LDH assesment. LDH is the enzyme responsible for the reversible conversion of pyruvate to lactate (Vassault, 1983) and is very important since we can use its value to identify stress conditions under low or no oxygen levels. In the specific case of *C. fluminea*, LDH is replaced by octopine dehydrogenase (ODH) as the terminal enzyme of anaerobic glycolysis (Livingstone *et al.,* 1990).

Esterases (EST) are a large family of enzymes that hydrolyses carboxylic esters, among other substrates (Garcia *et al.*, 2000). ChE belong to this enzyme family and are more prone to hydrolyze choline esters than other carboxylic esters as well as being inhibited by the carbamate eserine, therefore this can be used to distinguish them from other esterases (Mora *et al.*, 1999). Cholinesterases (ChE) are a common biomarker of neurotoxic effect and some pollutants are very specific to cholinesterases. These enzymes can be divided in true cholinesterases, such as acetylcholinesterase (AChE), and non-specific esterases or pseudocholinesterases (PChE) (Mora *et al.*, 1999). They differ from each other by the type of substrate they have more affinity to, translating into different levels of activity depending on the organism and tissue in cause (Mora *et al.*, 1999). Therefore, it is important to determine which kind is present as to avoid biased conclusions and assumptions regarding this particular biomarker (Mora *et al.*, 1999).

1.4. *Corbicula fluminea*

Corbicula fluminea (Müller, 1774) is a freshwater bivalve of Asian origin, considered one of the major non-native invasive species (NIS) of concern in aquatic ecosystems (Sousa *et al.*, 2008). According to the Convention on Biological Biodiversity (CBD), a NIS is an exotic species whose introduction and/or spread threats biological diversity (CBD, 2002).

Table 2 - General characteristics of *Corbicula fluminea* as an ecosystem engineer (adapted from Sousa *et al.,* 2008).

Effects on biodiversity	Effects on water quality	Effects on food availability
Can provide shelter and substrate for other species	Reduced eutrophication processes due to high filtration rates	*C. fluminea* can be a food resource for pelagic and benthic species
Can displace and/or reduce available habitat for other species	Increased water clarity due to high filtration rates	Competitor for benthic food resources with other species
Possible negative impacts on recruitment of other species caused by suspension and deposit feeding by *C. fluminea*	Specific environmental conditions causing massive mortalities can affect water quality	Limitation of planktonic food to other species

This bivalve, commonly known as Asian clam, was first recorded outside its original habitat in British Columbia, United States of America (USA) (Counts, 1981). Since then, this species has been spreading worldwide, with records of its presence on both Pacific and Atlantic Coasts of the USA, South America (Ituarte, 1994) and also in Europe (Mouthon, 1981). In Portugal, this species is also widespread through the tidal freshwater (TFW) of many rivers, in particular and of relevance for this study, those belonging to both Minho and Lima Rivers (Sousa *et al.*, 2006; Sousa *et al.*, 2008).

The introduction of a NIS may have various effects: it can go all the way from damages to the native biodiversity through impacts in human economy (Kolar & Lodge, 2001; Grosholz, 2002). In the particular case of *C. fluminea*, due to its characteristics as an ecosystem engineer (Table 2) it can result in various threats (Mooney *et al*. 2005; Hulme, 2006). But what makes *C. fluminea* such a successful NIS and its worldwide

establishment? Sousa *et al.* (2008) consider that, based on previous studies, what fuels the spread of this clam is its rapid growth, earlier sexual maturity, short life span, high fecundity and association with human activities.

Table 3 - *Corbicula fluminea* advantages for its use as a freshwater bioindicator species (data gathered from Doherty, 1990; Sousa *et al.*, 2006, Sousa *et al.,* 2008)

Spatial distribution	Wide spatial distribution, being a major component of several benthic communities in different regions of the world; Can be found either in pristine or polluted environments
Abundance	Very high abundance, due to its strong invasive dynamics
Laboratory maintenance	Easily maintained in laboratory conditions
Field studies	Can be transplanted into field conditions using caging procedures
Size	Adult's size allows dissection and separation of the main organs, allowing specific analysis
Contaminants	Ability to bioaccumulate and bioamplify various contaminants

In the particular case of the invasion of Minho and Lima rivers, and although this two rivers are in a short geographical distance of each other, plus their similarities in terms of hydrology and geology, previous studies highlight differences in both population structure and dynamics (Sousa *et al.* 2006; Sousa *et al.* 2008). The first scientific record of Minho river colonization by this species was published in 1989 (Araújo *et al.,* 1993). Since then, the population spread considerably being now a major component of the benthic

community, in terms of abundance and biomass (Sousa & Rufino, 2008). Lima had no record of this species until 2002, and seems to be restricted to a small area, at the upper estuary, with low abundance and biomass (Sousa *et al.* 2006).

Bivalves are no strangers to assessing biological impacts in aquatic environments, for example, mussels such as *Mytilus galloprovincialis* have been used as bioindicator organisms for Environmental Risk Assessment (ERA) (Porte *et al*., 2001) because of a series of characteristics that make them suitable for use in biomonitoring programmes (Lau & Wong, 2003). These features, among others (Table 3), are shared by *C. fluminea*, and give it an interesting standpoint regarding its use as a freshwater bioindicator species. As the two studied rivers show us two different population structure and dynamics (Sousa *et al.,* 2006, Sousa *et al.,* 2008), it would be of interest to assess if there are differences between these two populations, in terms of biomarkers indicative of oxidative stress, biotransformation, physiologic stress and alterations in the pathways of energy production.

1.5. Objectives and outline of the thesis

Considering the use of *C. fluminea* as a sentinel species in monitoring programmes based on biomarkers and the importance of reducing the bias that confounding factors may introduce in the interpretation of data from these studies, the objectives of this Thesis were to:

- characterize the ChE enzymes present in distinct tissues (adductor muscle, foot, gills and haemolymph) of *Corbicula fluminea* from the estuaries of Minho and Lima Rivers;
- study the seasonal variability of *Corbicula fluminea* EST, GST, CAT, GPx, GR, IDH and ODH enzymes and LPO levels in a reference site of the Minho River estuary providing the basis for their further use in more exhaustive environmental biomonitoring programmes.

The Thesis includes four chapters: Chapter I - General Introduction; Chapter II – Characterization of the ChE present in specific tissues of *Corbicula fluminea* (Mollusca: Bivalvia) from two estuaries of the NW Iberian coast; Chapter III – Biomonitoring and seasonal variability of *Corbicula fluminea* selected biomarkers in the Minho River estuary; and Chapter IV – Conclusions and Future Perspectives. In chapter I, an introduction to the contributions that Ecotoxicology may give to Forensic Medicine, to environmental monitoring, and to the species used as model is made, and the objectives and outline of

the Thesis are presented. In Chapter II, the characterization of ChE enzymes present in four tissues (adductor muscle, haemolymph, gills and feet) of *C. fluminea* was done. Chapter III corresponds to a biomonitoring study carried out for four seasons (Winter 2011, Spring 2012, Summer 2012 and Autumn 2012) with *C. fluminea* collected in a sampling site in the Minho River estuary. Finally, Chapter 4 corresponds to conclusions and future perspectives, where the main findings and contributions to the advance of knowledge of the work done are highlighted.

1.6. References

Araújo, R.; Moreno D.; Ramos, M.A. 1993. The asiatic clam *Corbicula fluminea* (Müller, 1774) (Bivalvia: Corbiculidae) in Europe. *American Malacological Bulletin*, *10*: 39-49.

Blokhina, O.; Virolainen, E.; Fagerstedt, K. V. 2003. Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Annals of Botany, 91:* 179-194.

Bucheli, T.; Fent, K. 1995. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems*. Critical Reviews in Environmental Science and Technology*, *25:* 201-268.

Carmichael, W.W.; Azevedo, S.M.F.O.; An, J.S., Molica, R.J.R; Jochimsen, E.M. 2001. Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins. *Environmental Health Perspectives*, *109:* 663–668.

Convention on Biological Biodiversity (CBD). 2002. Sixth Conference of the Parties, The Hague, the Netherlands, 7–19 April 2002: Decision VI/23: Alien species that threaten ecosystems, habitats or species to which is annexed guiding principles for the prevention, introduction and mitigation of impacts of alien species that threaten ecosystems, habitats or species.
Available at http://www.cbd.int/decision/cop/default.shtml?id=7197.

Counts, C.L. 1981. *Corbicula fluminea* (Bivalvia: Corbiculidea) in British Columbia. *Nautilus, 95:* 12-13.

Doherty, F.G. 1990. The asiatic clam, *Corbicula* spp, as a biological monitor in freshwater environments. *Environmental Monitoring and Assessment, 15:* 143-181.

França, G. V. 2005. *Fundamentos de Medicina Legal*. Guanabara Koogan, Rio de Janeiro.

Garcia, L. M.; Castro, B.; Guilhermino, L. 2000. Characterization of cholinesterase from guppy (*Poecilia reticulata*) muscle and its in vitro inhibition by environmental contaminants. *Biomarkers*, *5:* 274-284.

Grosholz, E. 2002. Ecological and evolutionary consequences of coastal invasions. *Trends in Ecology & Evolution*, *17:* 22-27.

Hill, E. F.; Fleming, W. J. 1982. Anticholinesterase poisoning of birds: Field monitoring and diagnosis of acute poisoning. *Environmental Toxicology and Chemistry, 1:* 27–38.

Hulme, P.E. 2006. Beyond control: wider implications for the management of biological invasions. *Journal of Applied Ecology*; *43:* 835–847.

Ituarte, C.F. 1994. *Corbicula* and *Neocorbicula* (Bivalvia: Corbiculidae) in the Paraná, Uruguay, and Rio de la Plata basins. *Nautilus*, 107: 129-135.

Kolar, C.S.; Lodge, D.M. 2001. Progress in invasion biology: predicting invaders. *Trends in Ecology & Evolution*, 16: 199-204.

Lau, P.; Wong, H. 2003. Effect of size tissue parts and location on six biochemical markers in the green-lipped mussel, *Perna viridis*, *Marine Pollution Bulletin*, 46: 1536-1572.

Lima, I.; Moreira, S. M.; Rendon-von Osten, J.;Soares, A. M.; Guilhermino, L. 2007. Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the North-western coast of Portugal. *Chemosphere*, *66:* 1230-1242.

Livingstone, D.R.; Stickle, W.B.; Kapper, M.A.; Wang, S.; Zurburg, W. 1990. Further studies on the phylogenetic distribuition of piruvate oxidoreductase activities. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, *97:* 661-666.

Markert, B.; Wünschmann, S.; Fränzle, S.; Wappelhorst, O.; Weckert, V.; Breulmann, G.; Djingova, R.; Herpin, U.; Lieth, H.; Schröder, W.; Siewers, U.; Steinnes, E.; Wolterbeek, B.; Zechmeister, H. 2008. On the road from biomonitoring to human health aspects - monitoring atmospheric heavy metal deposition by epiphytic/epigenetic plants: present status and future needs. *International Journal of Environment and Pollution*, *32:* 486–498.

Mooney, H.A.; Mack, R.N.; McNeely, J.A.; Neville, L.E.; Schei, P.J.; Waage, J.K. 2005. *Invasive Alien Species: A New Synthesis*. Island Press, Washington, DC.

Mora, P.; Fournier, D.; Narbonne, J. F. 1999. Cholinesterases from the marine mussels *Mytilus galloprovincialis* Lmk. and *M. edulis* L. and from the freshwater bivalve *Corbicula fluminea* Muller. *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology*, *122:* 353-361.

Mouthon, J. 1981. Sur la presence en France et au Portugal de Corbicula (Bivalvia, Corbiculidae) originaire d'Asie. *Basteria, 45*: 109-116.

Peakall, D. 1992. *Animal Biomarkers as Pollution Indicators*. Chapman and Hall, London.

Peakall, D.; Walker, C. 1994. The role of biomarkers in environmental assessment. *Ecotoxicology, 3:* 173-179.

Pimentel, D.; Andow, D.; Dyson-Hudson, R.; Gallahan, D.; Jacobson, S; Irish, M.; Kroop, S.; Moss, A.; Schreiner, I.; Shepard, M.; Thompson, T.; Vinzant, B. 1980. Environmental and social costs of pesticides: a preliminary assessment. *Oikos*, *34:* 126-140.

Porte, C.; Biosca, M.; Solé, M.; Albaigés, J. 2001. The integrated use of chemical analysis, cytochrome P450 and stress proteins in mussels to assess pollution along the Galician coast (NW Spain). *Environmental Pollution*, *112:* 261-268.

Sousa, R.; Rufino, M. 2008. Abiotic impacts on spatial and temporal distribution of *Corbicula fluminea* (Müller, 1774) in the River Minho Estuary, Portugal. *Aquatic Conservation: Marine and Freshwater Ecosystems, 110:* 98–110.

Sousa, R.; Antunes, C.; Guilhermino, L. 2006. Factors influencing the occurence and distribuition of Corbicula fluminea (Müller, 1774) in the River Lima estuary. *Annales de Limnologie - International Journal of Limnology*, *42:* 165-171.

Sousa, R.; Antunes, C.; Guilhermino, L. 2008. Ecology of the invasive Asian clam *Corbicula fluminea* (Müller, 1774) in aquatic ecosystems: an overview. *Annales de Limnologie - International Journal of Limnology*, *44:* 85-94.

Stegeman, J. J. 1985. Benzo[a]pyrene oxidation and microsomal enzyme activity in the mussel (*Mytilus edulis*) and other bivalve mollusc species from the Western North Atlantic. *Marine Biology*, *89:* 21-30.

Valavanidis, A.; Vlahogianni, T.; Dassenakis, M.; Scoullos, M. 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicology and Environmental Safety*, *64:* 178-89.

van der Oost, R.; Beyer, J.; Vermeulen, N. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology*, *13:* 57-149.

Vassault, A. 1983. M.O. Enzymes: oxireductases, transferases In: *Methods of Enzymatic Analysis*. Bergmeyer (Ed.), Academic Press, New York, 118–126

Vasseur, P.; Cossu-Leguille, C. 2006. Linking molecular interactions to consequent effects of persistent organic pollutants (POPs) upon populations. *Chemosphere*, *62:* 1033-42.

Walker, C.H.; Hopkin, S.P.; Sibly, R.M.; Peakall, D.B. 2006. *Principles of Ecotoxicology*. 3rd Edition, CRC Press, Boca Raton.

Winston, G. W.; Giulioz, R. T. D. 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquatic Toxicology, 19:* 137-161.

Chapter II – Characterization of the Cholinesterases Present in Specific Tissues of *Corbicula fluminea* (Mollusca: Bivalvia) From Two Estuaries of the NW Iberian Coast

Characterization of the cholinesterases present in specific tissues of *Corbicula fluminea* (Mollusca: Bivalvia) from two estuaries of the NW Iberian coast

Rocha, J.[1,2] and Guilhermino, L. [1,2]

[1] ICBAS - Institute of Biomedical Sciences of Abel Salazar, University of Porto, Department of Populations Studies, Laboratory of Ecotoxicology, Rua de Jorge Viterbo Ferreira, 228, 4050-313, Portugal

[2] CIIMAR – Interdisciplinary Centre of Marine and Environmental Research, Laboratory of Ecotoxicology and Ecology, University of Porto, Rua dos Bragas, 289, 4050-123 Porto, Portugal

2.1. Abstract

The objectives of the present study were: (i) to characterize the cholinesterase (ChE) enzymes present in different tissues (adductor muscle, gills, haemolymph and foot) of the Asian clam (*Corbicula fluminea*), an exotic invasive species in Europe; (ii) to investigate if populations from tidal freshwater areas of two estuaries (Minho and Lima Rivers, NW Portugal) with different contamination levels similar ChE enzymes; and (iii) if within the same population, environmental contamination may modulate the type of ChE present in specific tissues. Clams were collected in the two estuaries in one site in Minho Lima estuaries and in three different sites along an increasing gradient of environmental contamination towards the month of the estuary. A biochemical approach based on the Elman's technique, using different substrates (acetylthiocholine, butyrylthiocholine and propionylthiocholine) and selective inhibitors (eserine sulfate, BW284C51 and iso-OMPA) were used. In the adductor muscle and haemolymph, the results suggest the presence of one ChE with properties of both acetylcholinesterase and pseudocholinesterase (PChE) considering the typical vertebrate forms. In gills, a different form seems to be present with properties also similar to a intermediate form between vertebrate PChE and AChE. Finally, in foot tissue, the enzyme(s) present seem to be non-specific esterases. The pattern of enzymatic response was similar in the two populations. Overall, these results highlight the importance of using specific tissues rather than the whole body of *C. fluminea* in biomonitoring studies including ChE activity as biomarker, and the importance of enzymatic characterization.

Keywords: exotic invasive species, *Corbicula fluminea,* cholinesterase characterization, biomonitoring, AChE, ChE

2.2. Introduction

The Asian clam *Corbicula fluminea* is an exotic invasive species in Europe and in several other regions in the world. It is considered one of the most concerning non-native invasive species (NIS) in aquatic ecosystems and has been causing considerable economic and ecological problems in invaded ecosystems (Sousa *et al.*, 2008). Because it has been shown to be a good sentinel species (Doherty, 1990) and being an exotic invasive species their experimental use has limited ethical concerns (Doherty, 1990), it seems to be a convenient organisms for monitoring studies designed to assess ecosystem health among other purposes. However, before carrying out routine monitoring programs based on biomarkers, it is important to investigate the effects that confounding factors may have on the data to be obtained (van der Oost *et al.*, 2003).

Among the biomarkers that have been widely used in biomonitoring studies with mollusks, the activity of cholinesterase (ChE) enzymes has been having a highlighted position. It has been used in biomonitoring studies with several mollusk species, including marine (e.g. Dimitriadis *et al.*, 2012; Moreira & Guilhermino, 2001; Moreira *et al.*, 2004; Owen *et al.*, 2002; Tim-Tim et al., 2009) and freshwater (e.g. Mahamoud *et al.*, 2010; Valdez Domingos *et al.*, 2007; Wepner *et al.*, 2005). In general, the use of ChE as biomarkers is based on the inhibition of the enzymatic activity and considered indicative indicative of neurotoxicity because one of these enzymes, generally designed by acetylcholinesterase (AChE) based on the properties of vertebrate ChE, makes the degradation of the neurotransmitter acetylcholine in cholinergic synapses of the nervous system of both vertebrates and invertebrates. However, more than of ChE enzyme may be present in the tissues used, some of them have no known role in neurofunction, other enzymes, namely non-specific esterases may also contribute to the measured activity, and different enzymes may have different sensitivities towards environmental contaminants. For these reasons, it is important to make the characterization of the enzymes present in the species specific tissues to be used in biomonitoring studies (Kristoff *et al.*, 2006).

Esterases (EST) are a large enzyme family that hydrolyses carboxylic esters, among other substrates (Mora *et al.*, 1999). ChE belong to this enzyme family and are more prone to hydrolise choline esters than other carboxylic esters as well as being inhibited by the carbamate eserine, therefore this can be used to distinguish them from other esterases (Mora *et al.*, 1999). ChE include AChE and other enzymes known as pseudocholinesterases (PChE) (Thompson & Walker, 1994). AChE is known for its major

and important role in the function of the nervous system, hydrolyzing acetylcholine, the neurotransmitter in cholinergic synapses of the nervous system (Payne *et al.*, 1996; Thompson & Walker, 1994). As for PChE, this is a less specialized enzyme whose specific role is not yet known, although they appear to have a protective function by sequestering circulating organophosphate compounds and other anti-cholinesterase agents, decreasing their toxic effects on brain AChE (Kozlovskaya *et al.*, 1993; Witter, 1963). In vertebrates, these two enzymes can be distinguished by their behavior towards different substrates and inhibitors: AChE hydrolyzes preferably acetyltiocholine than other substrates, it is inhibited at high substrate concentrations, it is very sensitive to 1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284C51) and it is relatively insensitive to N,N´-diisopropylphosphorodiamic acid (iso-OMPA) (Xu & Bull, 1994). On the other hand, PChE prefers butirylthiocholine or propionylthicholine as substrate, it is not inhibited by high concentrations of substrate, it is very and low concentrations of iso-OMPA inhibit this enzyme, and it is relatively insensitive to BW284C51 (Xu & Bull, 1994). These characteristics are rather important as to characterize and localize the present forms in diferent tissues, species, or different tissues from the same species, as diferent forms can be present in different tissues. Although these characteristics might be valid for vertebrates and some invertebrates, some studies suggest that uncharacteristically ChE might not be able to be distinguished as either AChE or PChE as they can show characteristics from both enzyme types (Bocquene *et al.*, 1997; Liu *et al.*, 1994). Besides being different from one species to another, ChE also can differ in different tissues from the same species, as they are polymorphic in most species (Xu & Bull, 1994) its characterization must be done as to identify which type is present in the tissues one wants to study and also which tissue or tissues are best suited for ecotoxicological evaluation (Bocquene *et al.*, 1990; Garcia *et al.*, 2000; Hyne & Maher, 2003).

The characterization of *C. fluminea* ChE was done previously (Mora *et al.*, 1999; Ramos *et al.*, 2012), but only in whole body, never in different tissues from this bivalve. In biomonitoring studies, often a specific tissue is used to take advantage of different tissues for determining several biomarkers (Tim-Tim *et al.*, 2009, Lima *et al.,* 2007). Therefore, the characterization of the ChE present in the tissues generally used in biomonitoring studies with bivalves (adductor muscle, haemolymph, gills and foot) was made in *C. fluminea* from wild populations inhabiting tidal freshwater (TFW) areas of the estuaries of Minho and Lima Rivers (NW Iberian Peninsula) was made. Furthermore, the hypothesis that pollution may modulate the enzymes present was investigated.

2.3. Material and Methods

Animal sampling

Organisms were sampled in the TFW areas of Minho (M0; M1; M3) and Lima Rivers estuaries, in apparently differentially impacted sites by anthropogenic activities, and each sampling site had the following coordinates:

M0 (near Lapela, Friestas): N42º03'22.51'' W8º32'22.51''

M1 (near Barreiras, Friestas): N42º03'09.37'' W8º33'42.73''

M3 (near Lanhelas, Caminha): N41º54'41.25'' W8º47'36.59''

Lima (near Vila Mou) : N41º42'07.03'' W8º44'37.05''

Preparation of biological material

The sacrifice of animals was made after sampling, in the laboratory. Haemolymph, adductor muscle, gills and foot tissues were isolated on ice and removed to ice-cold phosphate buffer (0.1M, pH=7.2). Haemolymph was diluted in a proportion of 1:3 in cold phosphate buffer (0.1M, pH=7.2) and immediately stored at -80ºC. Then homogenates were prepared in the same buffer and using Ystral GmbH Dottingen homogenizer. All of the homogenizations were done on ice to prevent degradation. After homogenization, adductor muscle, gills and feet samples were centrifuged at 3300*g* using a Sigma Laboratory Centrifuge, model 3K30, for 3 minutes and the supernatant were stored at -80ºC.

Chemicals

All chemicals were purchased from Sigma-Aldrich (Germany), with the exception of the protein assay dye reagent that was bought from BioRad laboratories (Germany) as well as the phosphate buffer chemicals that were bought from Merck (Damstadt, Germany).

Substrate preferences

To determine the substrate preference of the enzymes present in each tissue, *in vitro* assays with different substrates (acetylthiocholine, butyrilthiocholine and propionylthiocholine) where done independently. For each substrate, an initial stock solution with the concentration of 767.52 mM was prepared in ultra-pure (u.p.) water; this solution was then serial diluted (1:2 v/v) in u.p. water to prepare 13 additional stock solutions. In each assay, a reaction buffer constituted by 0.5 mL DTNB (5,5'-Dithiobis-(2-Nitrobenzoic Acid)), 14.1 mL phosphate buffer (0.1M, pH=7.2) and 1.0 mL of corresponding substrate stock solution was prepared. In the assay, 0.250 mL of each

reaction was added to 0.05 mL of the corresponding biological sample, to obtain the final tested concentrations. Eight replicates were used per treatment. The activity was measured after five minutes as bellow described.

Specific inhibitors

To investigate the effects of selective inhibitors, an assay was done per inhibitor. For the assay with eserine sulphate by each ChE are inhibited but other esterases are not, a solution of eserine sulphate was prepared in u.p. water; this solution was then serial diluted 1:2 (v/v) with u.p. water; thus, six stock solutions with the following concentrations were obtained: 20, 10, 5, 2.5, 1.25 and 0.675 mM; from each solution, 0.005 mL were added to 0.495 mL of biological sample, and controls were incubated with 0.50 mL of water, thus the following concentrations of eserine sulphate were tested: 0, 6.25, 12.5, 25, 50, 100 and 200 µM; eight replicates were used per treatment and the activity was measured after an incubation period of 45 minutes at 25ºC. For BW284C51, selective inhibitor of AChE in mammals, a similar procedure was done but for this compound an additional concentration (400 µM) was tested. Finally, a similar procedure was followed in the assay with iso-OMPA but the compound was dissolved in ethanol due to its limited solubility in water, the concentrations of the stock solutions were higher (50 to 800 mM) and thus the final concentrations tested were: 0, 0.5, 1, 2, 4 and 8 mM.

Enzyme activity

ChE activity was determined in triplicate by the Ellman method (Ellman *et al.*, 1961) adapted to microplate (Guilhermino *et al.*, 1996). In short, in each assay well, 0.250 mL of reaction buffer (described above in *Substrate preferences*) was added to 0.05 mL of the corresponding biological sample (or u.p. water, for controls) and then activity was measured at 412 nm for five minutes, at 25ºC. The concentration of protein was determinate in triplicates by the Bradford method (Bradford, 1976) adapted to microplate, using bovine-γ-globuline as standard. A microplate reader (SpectraMax M2e) was used for both the analysis (enzymatic activity and protein concentration). The enzymatic activity was expressed as Units (U) per mg of protein, 1 U being a nanomole of substrate hydrolysed by minute per mg of protein.

Data analysis

All data analysis were performed using SPSS Statistics 21.0© software package. Test of homogeneity of variances (Levene Statistic) was performed to assess the homogeneity of variances. Normal distribution was assessed by the Kolmogorov–Smirnov test. For each assay of the selective inhibitors, one-way analysis of variance (ANOVA) was

performed to compare different treatments. When significant differences among treatments were found, the Dunnett's test was used to assess differences between control+solvent and each of the inhibitors concentrations. The significance level was 0.05.

2.4. Results and Discussion

Adductor muscle

Figure 1 shows the effects of eserine sulphate on the enzymatic activity of the adductor muscle of *C. fluminea* from different sites in the TFW areas of the Minho estuary (A) and from one site in the Lima estuary (B). In all cases, a significant (M0: $F_{6, 55} = 270.690$, $p < 0.05$; M1: $F_{6, 55} = 243.005$, $p < 0.05$; M3: $F_{6, 55} = 372.050$, $p < 0.05$; Lima: $F_{6, 55} = 287.413$, $p < 0.05$) almost complete inhibition of enzymatic activity (more than 95% of inhibition, Table 1) was observed in the range of concentrations tested (6.25 to 200 µmolar) indicating that it is an activity from ChE and not from other EST. In the range of concentrations tested, the substrate hydrolysed at the highest rate was propionylthiocholine (Figure 2), then acetylthiocholine and finally a very low activity was when butyrylthiocholine was used. The only exception to this trend being for samples of M3, where acetylthiocholine passed propionylthiocholine by a small difference (Figure 2(C)) The enzymatic activity was strongly inhibited by both BW284C1 (M0: $F_{7, 63} = 343.602$, $p < 0.05$; M1: $F_{7, 63} = 155.946$, $p < 0.05$; M3: $F_{7, 63} = 75.006$, $p < 0.05$; Lima: $F_{7, 63} = 91.472$, $p < 0.05$), a selective inhibitor of vertebrate AChE, and iso-OMPA (M0: $F_{6, 55} = 9.824$, $p < 0.05$; M1: $F_{6, 55} = 10.019$, $p < 0.05$; M3: $F_{6, 55} = 21.086$, $p < 0.05$; Lima: $F_{6, 55} = 8.297$, $p < 0.05$), a selective inhibitor of vertebrate PChE (in this case in a dose-response way), in the µmolar and mmolar range, respectively. These evidences suggest that the enzymatic activity measured is from a ChE with properties of both AChE and PChE considering the typical properties of these enzymes in vertebrates. The activity of soluble ChE determined in *C. fluminea* adductor muscle using the determinations made in control treatments of all the assays with selective inhibitors using acetylthiocholine as substrate in a concentration of 48 Mm (concentration in the reaction volume) was 8,076 ± 2,996 nanomol/min/mg protein (mean ± DP). The adductor muscle of bivalves is responsible for opening of the valves, a mechanism under nervous system control (Corsi *et al.*, 2004) that is also a most important function for the survival and performance of the animal. It is easy to isolate and in the case of *C. fluminea* has ChE activity in levels that can be accurately measured providing reproducible results (Figure 1). For these reasons is a very convenient tissue for measuring ChE activity in this species.

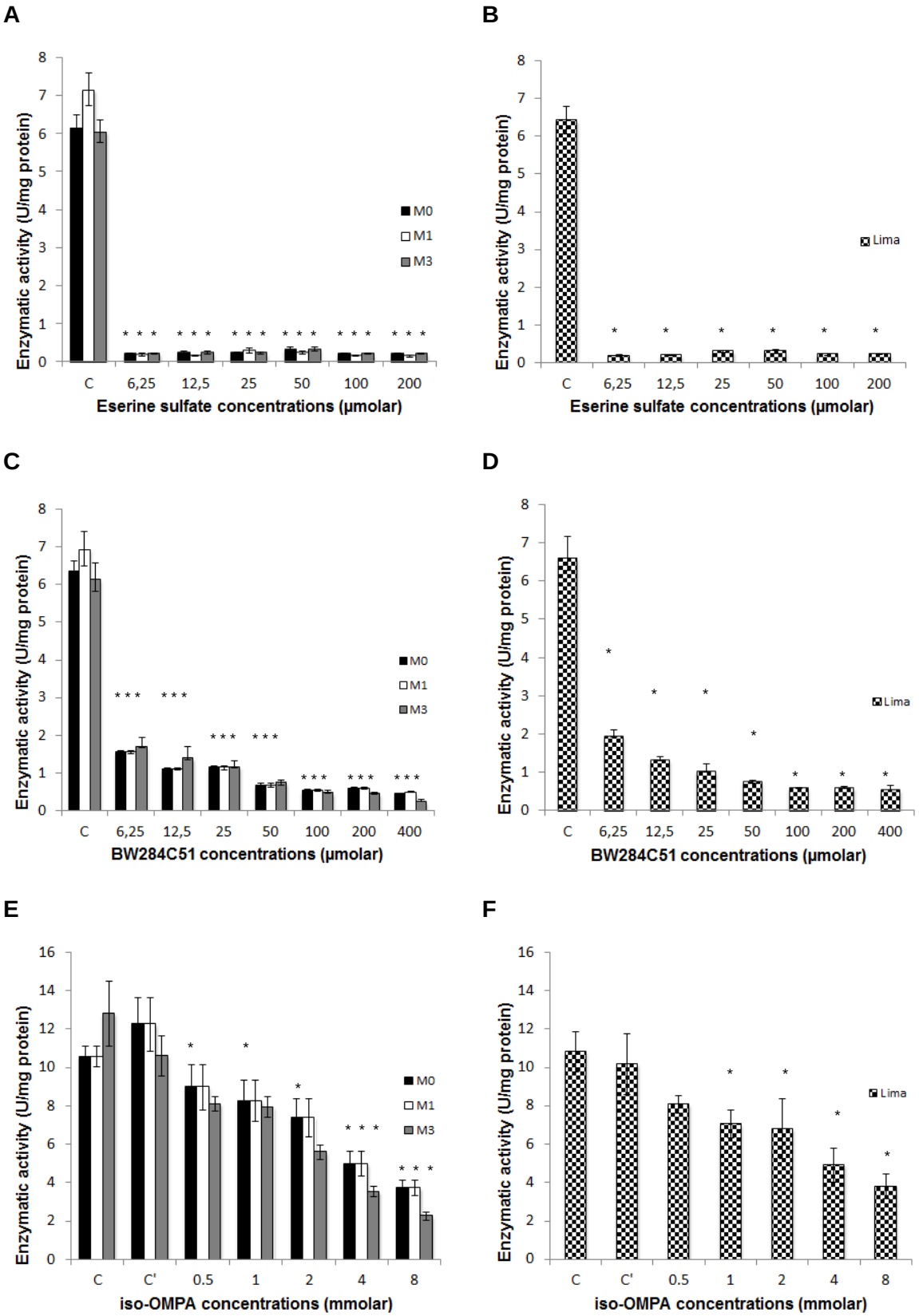

Figure 1 - Effect of specific inhibitors on the cholinesterasic activity determined in the adductor muscle of *Corbicula fluminea*. Values are the mean of eight replicates and corresponding standard error bars. The mean and standard deviation of activity for the adductor muscle determined from all the control treatments was 8,076 ± 2,996 nanomol/min/mg protein. A and B correspond to the effect of eserine sulphate on both Minho and Lima sampling sites, respectively. C and D correspond to the effect of BW284C51 on both Minho and Lima sampling sites, respectively. E and F correspond to the effect of iso-OMPA on both Minho and Lima sampling sites, respectively. C corresponds to control samples and C' to control with ethanol samples. * Indicates statistically significant differences ($p < 0.05$) from the control (C).

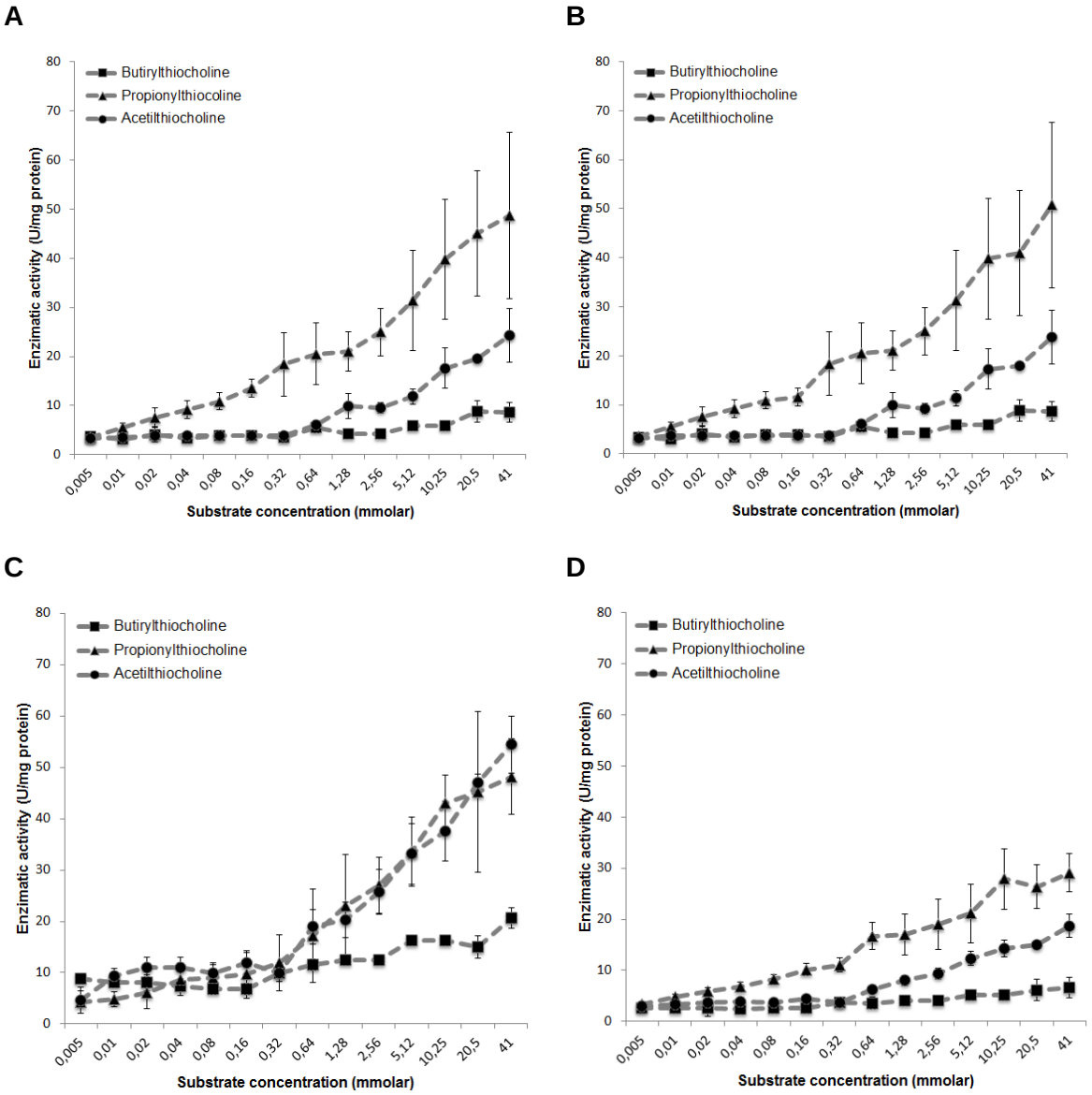

Figure 2 - Cholinesterasic activity determined in the adductor muscle of *Corbicula fluminea* as a function of acetylthiocholine, propionylthiocholine, butirylthiocholine concentrations. Values are the mean of eight replicates and corresponding standard error bars. The letters A, B, C and D correspond to the different sampling sites, M0, M1, M3 and Lima, respectively.

Table 1 - Percentage (%) of inhibition of enzyme activity determined in adductor muscle of *Corbicula fluminea* using eserine sulphate as an inhibitor, with respective standard error (S.E.M).

Eserine sulphate concentration (µM)	% inhibition of activity with eserine sulphate								
	M0			M1			M3			Lima		
6.25	96.35	±	5.80	97.40	±	15.84	96.30	±	5.79	97.00	±	11.75
12.5	96.18	±	13.07	97.84	±	9.86	96.13	±	13.06	96.64	±	10.40
25	96.26	±	7.76	96.02	±	24.26	96.21	±	7.75	95.12	±	8.28
50	94.61	±	18.02	96.75	±	15.55	94.54	±	18.01	95.08	±	17.02
100	96.60	±	3.97	97.86	±	12.46	96.55	±	3.97	96.41	±	8.91
200	96.66	±	5.10	97.91	±	13.96	96.61	±	5.10	96.30	±	7.42

Table 2 – Percentage of inhibition of enzyme activity determined in adductor muscle of *Corbicula fluminea* using BW284C51 as an inhibitor, with respective standard error (S.E.M).

BW284C52 concentration (µM)	% inhibition of activity with BW284C51								
	M0			M1			M3			Lima		
6.25	75.64	±	2.39	77.66	±	2.45	72.32	±	10.47	70.67	±	5.86
12.5	82.61	±	3.33	84.05	±	3.39	77.13	±	16.51	80.09	±	5.90
25	81.95	±	2.81	83.44	±	2.86	80.94	±	10.40	84.41	±	14.93
50	89.39	±	7.41	90.27	±	7.49	87.68	±	7.56	88.48	±	3.84
100	91.63	±	5.50	92.32	±	5.55	92.19	±	11.90	91.09	±	3.69
200	90.79	±	6.46	91.55	±	6.51	92.70	±	11.28	91.03	±	6.71
400	92.79	±	4.24	92.89	±	7.81	95.97	±	23.15	91.82	±	18.82

Table 3 - Percentage of inhibition of enzyme activity determined in adductor muscle of *Corbicula fluminea* using iso-OMPA as an inhibitor, with respective standard error (S.E.M.). C' corresponds to control with ethanol samples.

iso-OMPA concentration (mM)	% inhibition of activity with iso-OMPA			
	M0	M1	M3	Lima
C'	0 ± 1.66	0 ± 1.80	17.11 ± 1.71	5.87 ± 0.91
0.5	15.36 ± 1.98	15.13 ± 1.98	36.78 ± 1.80	25.39 ± 1.44
1	22.45 ± 2.91	21.83 ± 2.90	38.13 ± 2.57	34.77 ± 3.59
2	28.04 ± 3.59	30.02 ± 4.04	56.35 ± 3.87	37.02 ± 8.37
4	52.07 ± 6.02	52.87 ± 6.91	72.48 ± 5.47	54.44 ± 9.77
8	64.36 ± 8.63	64.82 ± 6.93	82.36 ± 7.41	64.92 ± 11.00

Haemolymph

Figure 3 shows the effects of eserine sulphate on the enzymatic activity of the haemolymph of *C. fluminea* from different sites in the TFW areas of the Minho estuary (A) and from one site in the Lima estuary (B). In all cases, a significant (M0: $F_{6, 55} = 203.538$, $p < 0.05$; M1: $F_{6, 55} = 246.138$, $p < 0.05$; M3: $F_{6, 55} = 603.261$, $p < 0.05$; Lima: $F_{6, 55} = 413.932$, $p < 0.05$) almost complete inhibition of enzymatic activity (more than 96% of inhibition, Table 4) was observed at all concentrations tested (6.25 to 200 µmolar range) indicating that it is an activity from ChE and not from other esterases. In the range of concentrations tested, the substrate hydrolysed at the highest rate was acetylthiocholine (Figure 4), then propionylthiocholine and finally a very low activity was achivied when butyrylthiocholine was substrate. The enzymatic activity was strongly inhibited by both BW284C1 (M0: $F_{7, 63} = 1492.388$, $p < 0.05$; M1: $F_{7, 63} = 1682.257$, $p < 0.05$; M3: $F_{7, 63} = 194.164$, $p < 0.05$; Lima: $F_{7, 63} = 328.437$, $p < 0.05$), a selective inhibitor of vertebrate AChE, and inhibited in some extent by iso-OMPA at the 2, 4 and 8 mmolar concentrations (M0: $F_{6, 55} = 31.813$, $p < 0.05$; M1: $F_{6, 55} = 23.776$, $p < 0.05$; M3: $F_{6, 55} = 26.541$, $p < 0.05$; Lima: $F_{6, 55} = 34.682$, $p < 0.05$), a selective inhibitor of vertebrate PChE (in this case in a dose-response way), in the µmolar and mmolar range, respectively. These evidences suggest that the enzymatic activity measured is from a ChE with properties of both AChE and PChE considering the typical properties of these enzymes in vertebrates.

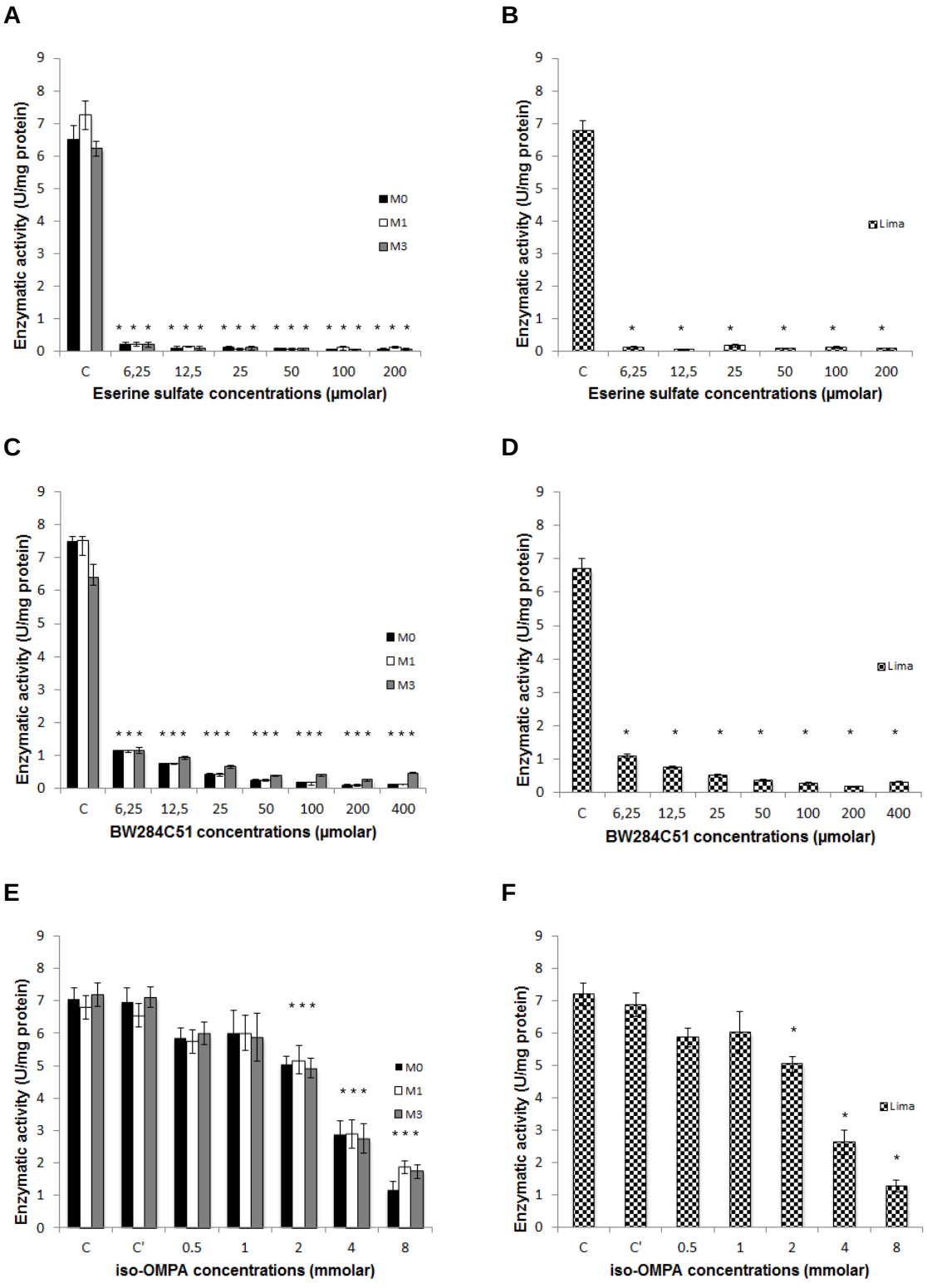

Figure 3 - Effect of specific inhibitors on the cholinesterasic activity determined in the haemolymph of *Corbicula fluminea*. Values are the mean of eight replicates and corresponding standard error bars. The mean and standard deviation of activity for the adductor muscle determined from all the control treatments was 6.965 ± 1.017 nanomol/min/mg protein. A and B correspond to the effect of eserine sulphate on both Minho and Lima sampling sites, respectively. C and D correspond to the effect of BW284C51 on both Minho and Lima sampling sites, respectively. E and F correspond to the effect of iso-OMPA on both Minho and Lima sampling sites, respectively. C corresponds to control samples and C' to control with ethanol samples. * Indicates statistically significant differences ($p < 0.05$) from the control (C).

The activity of soluble ChE determined in *C. fluminea* haemolymph using the determinations made in control treatments of all the assays with selective inhibitors using acetylthiocholine as substrate in a concentration of 48 mM(concentration in the reaction volume) was 6.965 ± 1,017 nanomol/min/mg protein (mean ± DP). Haemolymph is easy to isolate and in the case of *C. fluminea* has ChE activity in levels that can be accurately measured providing reproducible results (Figure 3). For these reasons is a very convenient tissue for measuring ChE activity in this species.

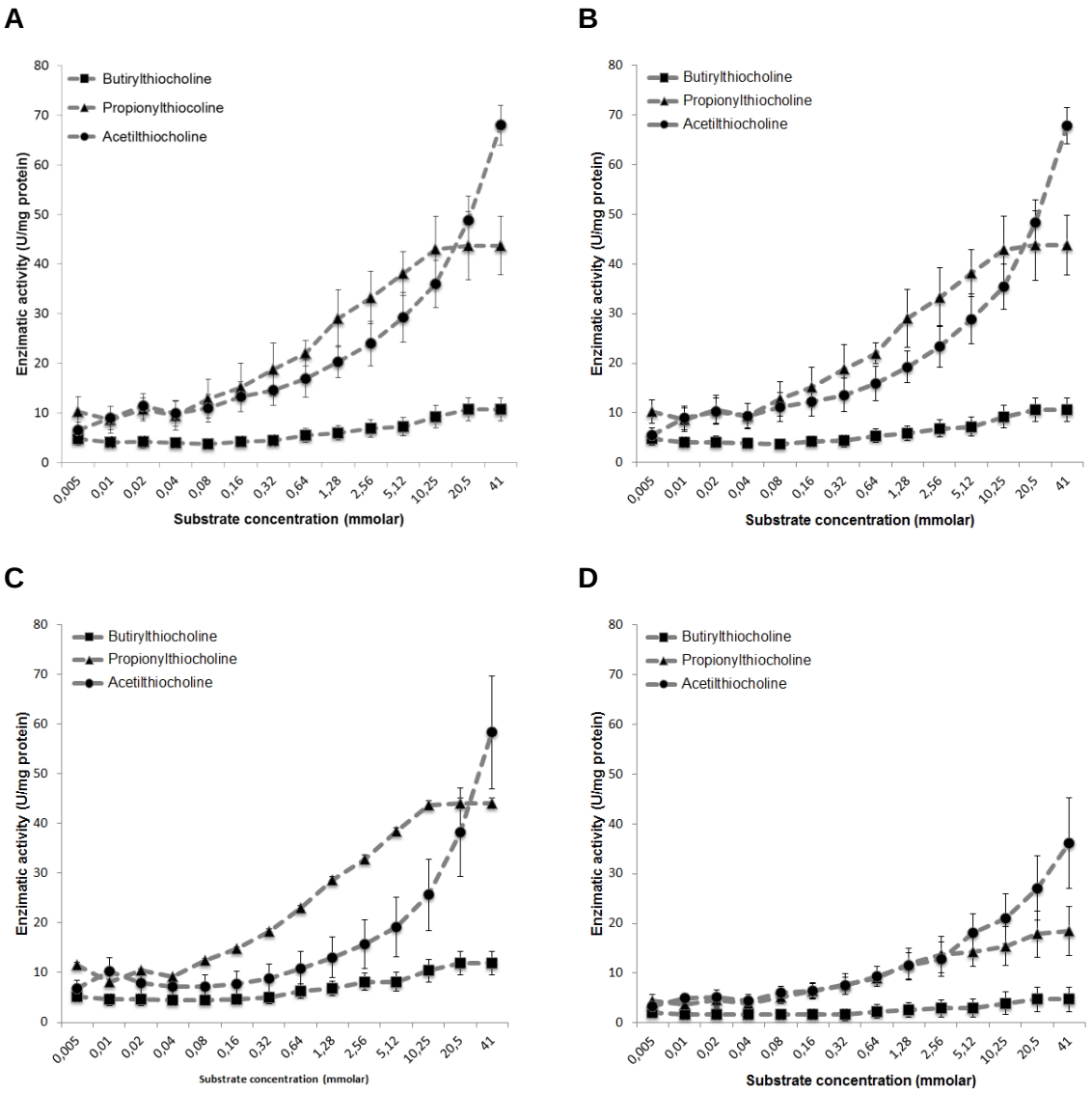

Figure 4 - Cholinesterasic activity determined in the haemolymph of *Corbicula fluminea* as a function of acetylthiocholine, propionylthiocholine, butirylthiocholine concentrations Values are the mean of eight replicates and corresponding standard error bars. The letters A, B, C and D correspond to the different sampling sites, M0, M1, M3 and Lima, respectively.

Table 4 - Percentage of inhibition of enzyme activity determined in haemolymph of *Corbicula fluminea* using eserine sulphate as an inhibitor, with respective standard error (S.E.M.)

Eserine sulphate concentration (µM)	% inhibition of activity with eserine sulphate			
	M0	M1	M3	Lima
6.25	96.88 ± 33.37	97.00 ± 25.51	96.75 ± 33.32	98.23 ± 31.84
12.5	98.49 ± 51.20	97.92 ± 11.42	98.43 ± 51.16	99.31 ± 28.04
25	98.26 ± 49.64	99.06 ± 45.19	98.19 ± 49.61	97.26 ± 27.36
50	98.77 ± 32.35	99.06 ± 29.33	98.72 ± 32.34	98.72 ± 28.75
100	99.02 ± 36.36	98.54 ± 53.71	98.98 ± 36.35	98.15 ± 27.53
200	99.02 ± 43.65	98.14 ± 26.79	98.98 ± 43.63	98.89 ± 23.32

Table 5 - Percentage of inhibition of enzyme activity determined in haemolymph of *Corbicula fluminea* using BW284C51 as an inhibitor, with respective standard error (S.E.M.)

BW284C52 concentration (µM)	% inhibition of activity with BW284C51			
	M0	M1	M3	Lima
6.25	80.57 ± 5.90	84.69 ± 1.56	82.03 ± 7.07	83.67 ± 4.98
12.5	89.96 ± 2.55	90.10 ± 3.58	85.18 ± 3.86	88.94 ± 6.27
25	94.74 ± 13.86	94.52 ± 13.12	89.42 ± 5.55	92.15 ± 3.46
50	96.68 ± 17.34	96.78 ± 19.54	93.91 ± 3.35	94.46 ± 5.94
100	97.84 ± 14.57	97.75 ± 12.97	93.63 ± 2.86	96.01 ± 12.66
200	98.51 ± 32.32	98.65 ± 29.17	95.94 ± 14.21	97.52 ± 23.69
400	98.54 ± 22.79	98.47 ± 23.75	92.79 ± 5.27	95.38 ± 8.31

Table 6 - Percentage of inhibition of enzyme activity determined in haemolymph of *Corbicula fluminea* using iso-OMPA as an inhibitor, with respective standard error (S.E.M.). C' corresponds to control with ethanol samples.

iso-OMPA concentration (mM)	% inhibition of activity with iso-OMPA											
	M0			M1			M3			Lima		
C'	1.10	±	0.06	3.75	±	0.21	1.08	±	0.05	4.55	±	0.24
0.5	16.98	±	0.81	15.60	±	0.96	16.69	±	0.97	18.43	±	0.88
1	15.04	±	1.73	11.74	±	1.05	18.26	±	2.30	16.52	±	1.78
2	28.64	±	1.37	24.03	±	2.04	31.62	±	1.92	29.88	±	1.43
4	59.28	±	8.67	57.70	±	8.73	61.73	±	10.15	63.46	±	9.35
8	83.42	±	18.60	72.66	±	7.74	75.88	±	9.62	82.20	±	10.97

Gills

Figure 5 shows the effects of eserine sulphate on the enzymatic activity of the gills of *C. fluminea* from different sites in the TFW areas of the Minho estuary (A) and from one site in the Lima estuary (B). In all cases, a significant (M0: $F_{6, 55} = 203.538$, $p < 0.05$; M1: $F_{6, 55} = 246.138$, $p < 0.05$; M3: $F_{6, 55} = 603.261$, $p < 0.05$; Lima: $F_{6, 55} = 413.932$, $p < 0.05$) inhibition of enzymatic activity (more than 60% of inhibition, Table 7) was observed at all concentrations tested (6.25 to 200 µmolar range) indicating that there is activity from ChE. The activity of soluble ChE determined in *C. fluminea* gills using the determinations made in control treatments of all the assays with selective inhibitors using acetylthiocholine as substrate in a concentration of 48 mM(concentration in the reaction volume) was 0,398 ± 0,164 nanomol/min/mg protein (mean ± DP). In the range of concentrations tested, the substrate hydrolysed at the highest rate was propionylthiocholine (Figure 6), then closely followed by acetylthiocholine and finally a very low activity was achivied when butyrylthiocholine was substrate. The results regarding inhibition towards specific inhibitors was not conclusive, as there was no found significant inhibition by BW284C51, and significant inhibition by iso-OMPA was only found in two of the four sampling sites, and only for the concentrations of 2, 4 and 8 mM (for M3 sampling site, M3: $F_{6, 55} = 6.942$ $p < 0.05$) and 4 and 8 mM (for Lima sampling site, Lima: $F_{6, 55} = 5.697$, $p < 0.05$). These evidences suggest that a different form seems to be present with properties similar to an intermediate form between vertebrate PChE and AChE, but could not be confirmed. For this reason, this is apparently not a very convenient tissue for measuring ChE activity in this species.

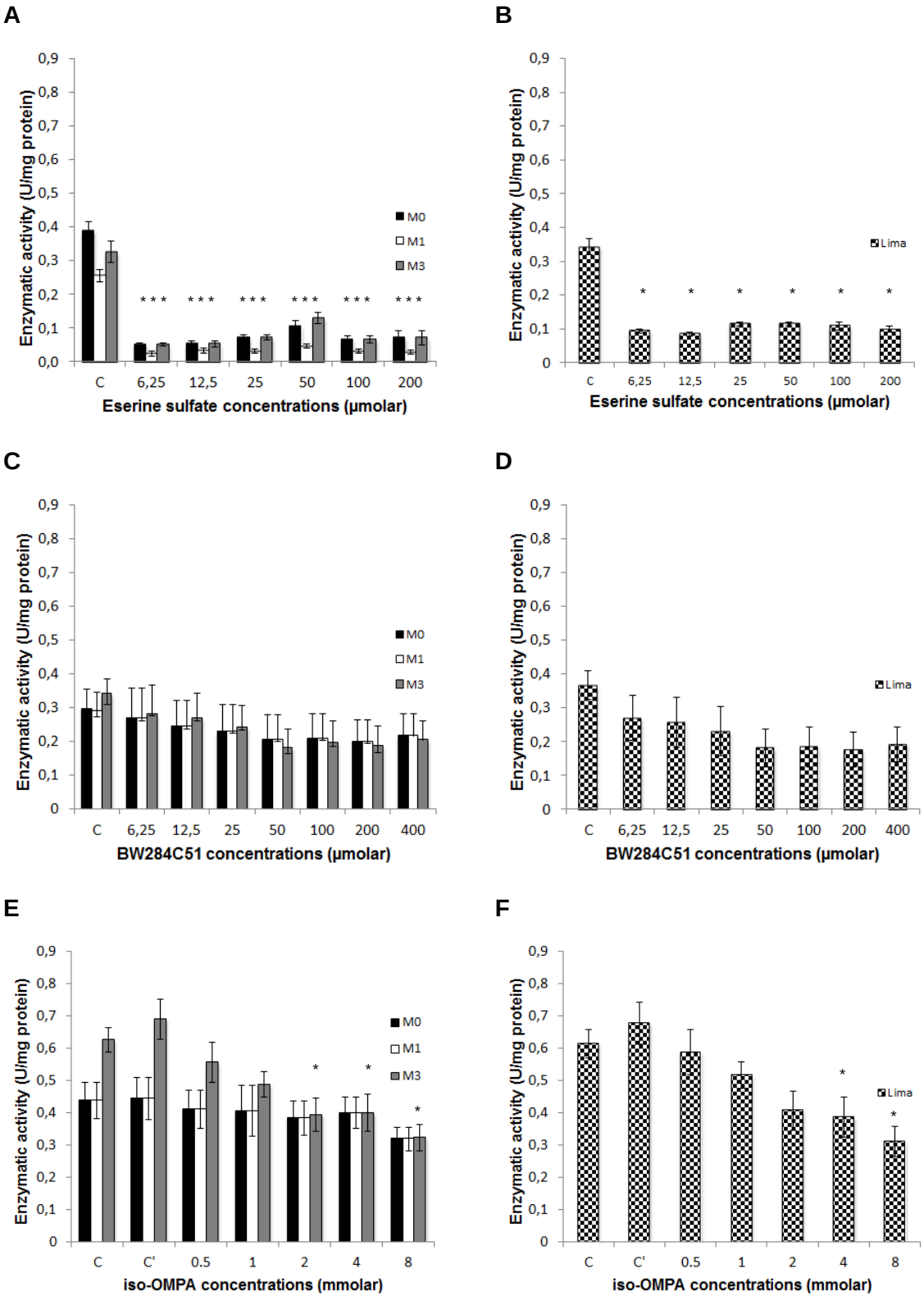

Figure 5 - Effect of specific inhibitors on the cholinesterasic activity determined in the gills of *Corbicula fluminea*. Values are the mean of eight replicates and corresponding standard error bars. The mean and standard deviation of activity for the adductor muscle determined from all the control treatments was 0.398 ± 0.164. A and B correspond to the effect of eserine sulphate on both Minho and Lima sampling sites, respectively. C and D correspond to the effect of BW284C51 on both Minho and Lima sampling sites, respectively. E and F correspond to the effect of iso-OMPA on both Minho and Lima sampling sites, respectively. C corresponds to control samples and C' to control with ethanol samples. * Indicates statistically significant differences ($p < 0.05$) from the control (C).

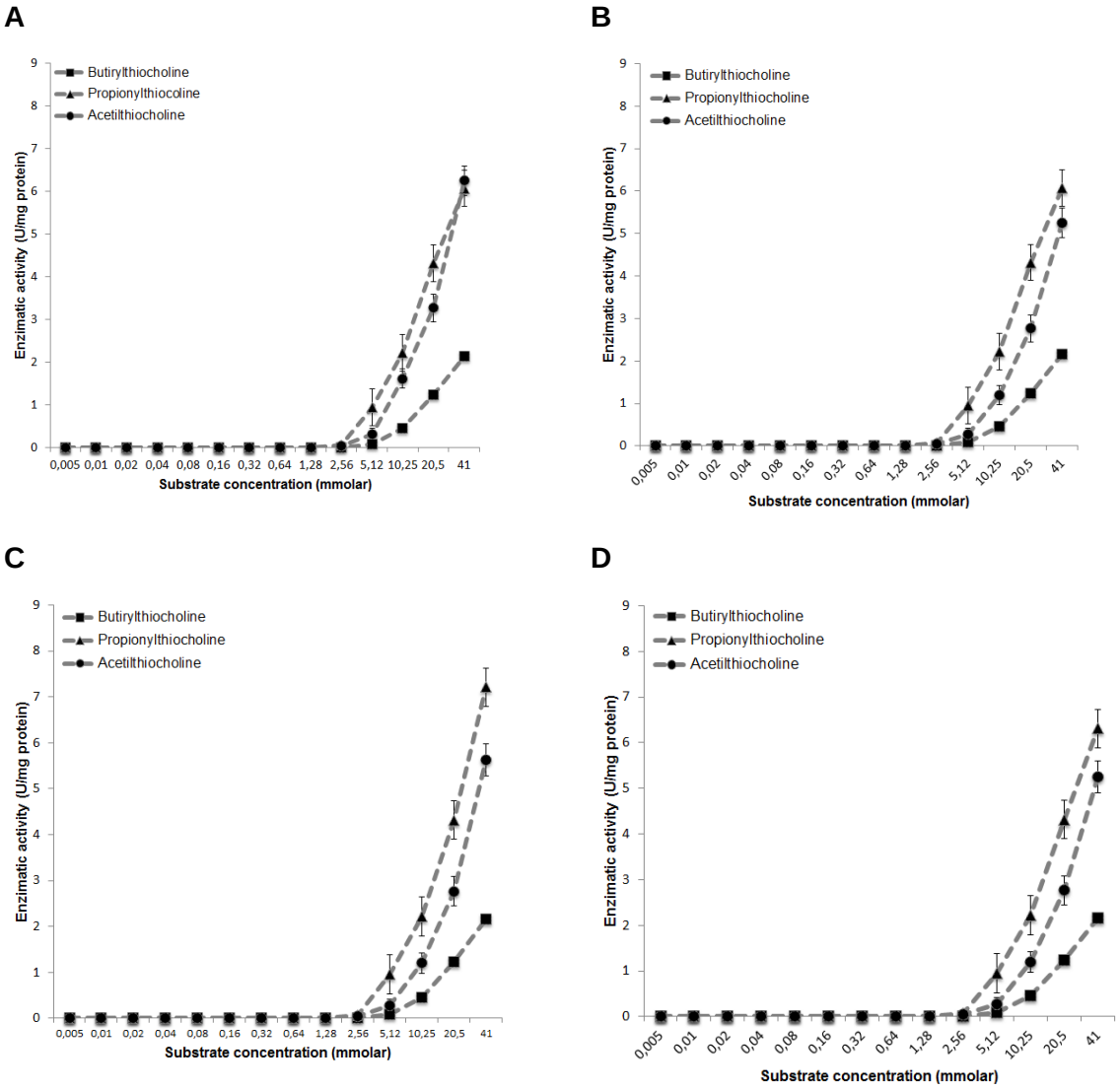

Figure 6 - Cholinesterasic activity determined in the gills of *Corbicula fluminea* as a function of acetylthiocholine, propionylthiocholine, butirylthiocholine concentrations Values are the mean of eight replicates and corresponding standard error bars. The letters A, B, C and D correspond to the different sampling sites, M0, M1, M3 and Lima, respectively.

Table 7 – Percentage of inhibition of enzyme activity determined in gills of *Corbicula fluminea* using eserine sulphate as an inhibitor, with respective standard error (S.E.M.)

Eserine sulphate concentration (µM)	% inhibition of activity with eserine sulphate											
	M0			M1			M3			Lima		
6.25	86.84	±	7.94	90.54	±	24.71	84.25	±	7.70	72.41	±	4.74
12.5	86.42	±	13.75	86.82	±	19.86	83.75	±	13.32	75.11	±	5.69
25	81.64	±	8.24	87.90	±	18.18	78.03	±	7.87	66.39	±	3.68
50	72.93	±	12.02	81.74	±	12.49	59.93	±	7.62	66.39	±	3.68
100	82.76	±	13.04	87.73	±	16.84	79.37	±	12.51	68.31	±	7.40
200	81.43	±	23.85	89.08	±	20.92	77.78	±	22.78	71.10	±	6.91

Table 8 – Percentage of inhibition of enzyme activity determined in gills of *Corbicula fluminea* using BW284C51 as an inhibitor, with respective standard error (S.E.M.)

BW284C52 concentration (µM)	% inhibition of activity with BW284C51											
	M0			M1			M3			Lima		
6.25	9.43	±	3.10	7.62	±	2.51	17.50	±	5.34	26.26	±	6.49
12.5	17.96	±	5.69	16.33	±	5.17	21.26	±	5.86	30.05	±	8.75
25	22.47	±	7.63	20.93	±	7.11	28.85	±	7.73	37.12	±	11.79
50	30.29	±	10.62	28.90	±	10.13	46.65	±	13.99	50.29	±	15.54
100	29.38	±	10.18	27.98	±	9.69	42.20	±	13.95	49.56	±	15.95
200	32.29	±	10.30	30.94	±	9.87	44.72	±	13.47	51.91	±	15.17
400	26.93	±	8.11	25.48	±	7.67	40.06	±	11.36	47.57	±	13.03

Table 9 - Percentage of inhibition of enzyme activity determined in gills of *Corbicula fluminea* using iso-OMPA as an inhibitor, with respective standard error (S.E.). C' corresponds to control with ethanol samples

iso-OMPA concentration (mM)	% inhibition of activity with iso-OMPA											
	M0			M1			M3			Lima		
C'	0.42	±	0.06	0	±	0.17	0	±	0.90	0	±	1.00
0.5	10.80	±	1.45	6.35	±	0.91	11.12	±	1.23	4.27	±	0.49
1	10.72	±	2.05	7.39	±	1.43	22.14	±	1.80	15.99	±	1.28
2	11.65	±	1.65	12.55	±	1.73	37.05	±	4.85	33.73	±	5.08
4	20.73	±	2.91	8.90	±	1.09	36.23	±	5.26	36.97	±	5.95
8	31.48	±	2.93	27.33	±	3.05	48.44	±	6.21	49.43	±	7.44

Foot tissue

Figure 7 shows the effects of eserine sulphate on the enzymatic activity of the foot tissue of *C. fluminea* from different sites in the TFW areas of the Minho estuary (A) and from one site in the Lima estuary (B). The only sampling site with significant, although not extensive, inhibition was M0, with all other sampling sites showing no significant inhibition (M0: $F_{6, 55} = 3.043$, $p < 0.05$; M1: $F_{6, 55} = 1.205$, $p < 0.05$; M3: $F_{6, 55} = 1.649$, $p < 0.05$; Lima: $F_{6, 55} = 1.145$, $p < 0.05$). This indicates that the esterases present in this tissue cannot be confirmed as ChE, but only as EST, although they can hydrolyse acetylthiocholine to some extent, and this is the substrate hydrolysed at the fastest rate (Figure 8), followed closely by propyonilthiocholine and a very low activity towards butyrylthiocholine. The activity of soluble EST determined in *C. fluminea* gills using the determinations made in control treatments of all the assays with selective inhibitors using acetylthiocholine as substrate in a concentration of 48 mM(concentration in the reaction volume) was 0,494 ± 0,191 nanomol/min/mg protein (mean ± DP). These evidences suggest that in this tissue the esterases present are not specifically of the ChE type. For this reason, this is not a very convenient tissue for measuring ChE activity in this species, but could possibly be used for measuring non-specific EST activity.

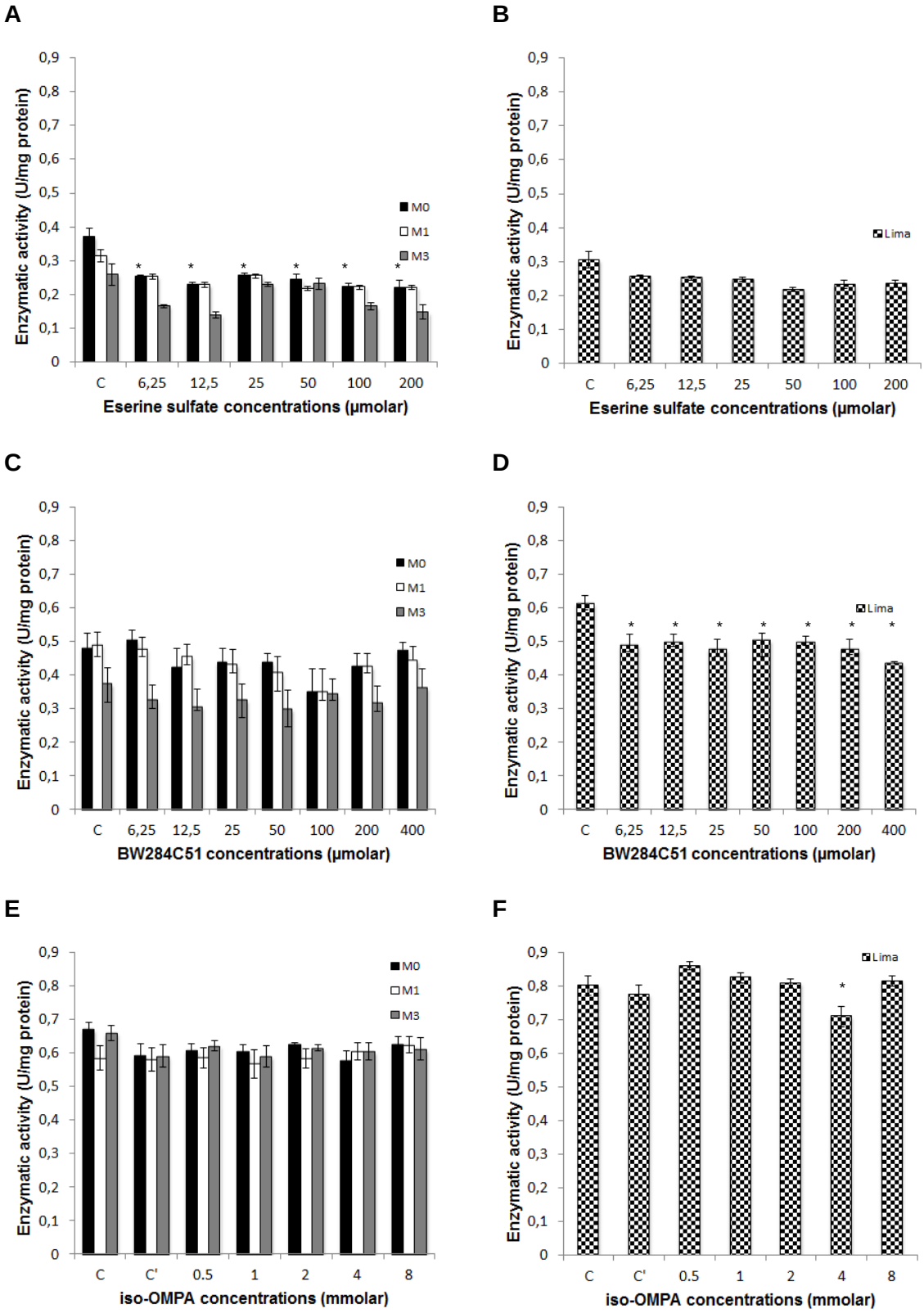

Figure 7 - Effect of specific inhibitors on the cholinesterasic activity determined in the foot tissue of *Corbicula fluminea*. Values are the mean of eight replicates and corresponding standard error bars. The mean and standard deviation of activity for the adductor muscle determined from all the control treatments was 0.494 ± 0.191. A and B correspond to the effect of eserine sulphate on both Minho and Lima sampling sites, respectively. C and D correspond to the effect of BW284C51 on both Minho and Lima sampling sites, respectively. E and F correspond to the effect of iso-OMPA on both Minho and Lima sampling sites, respectively. C corresponds to control samples and C' to control with ethanol samples. * Indicates statistically significant differences ($p < 0.05$) from the control (C).

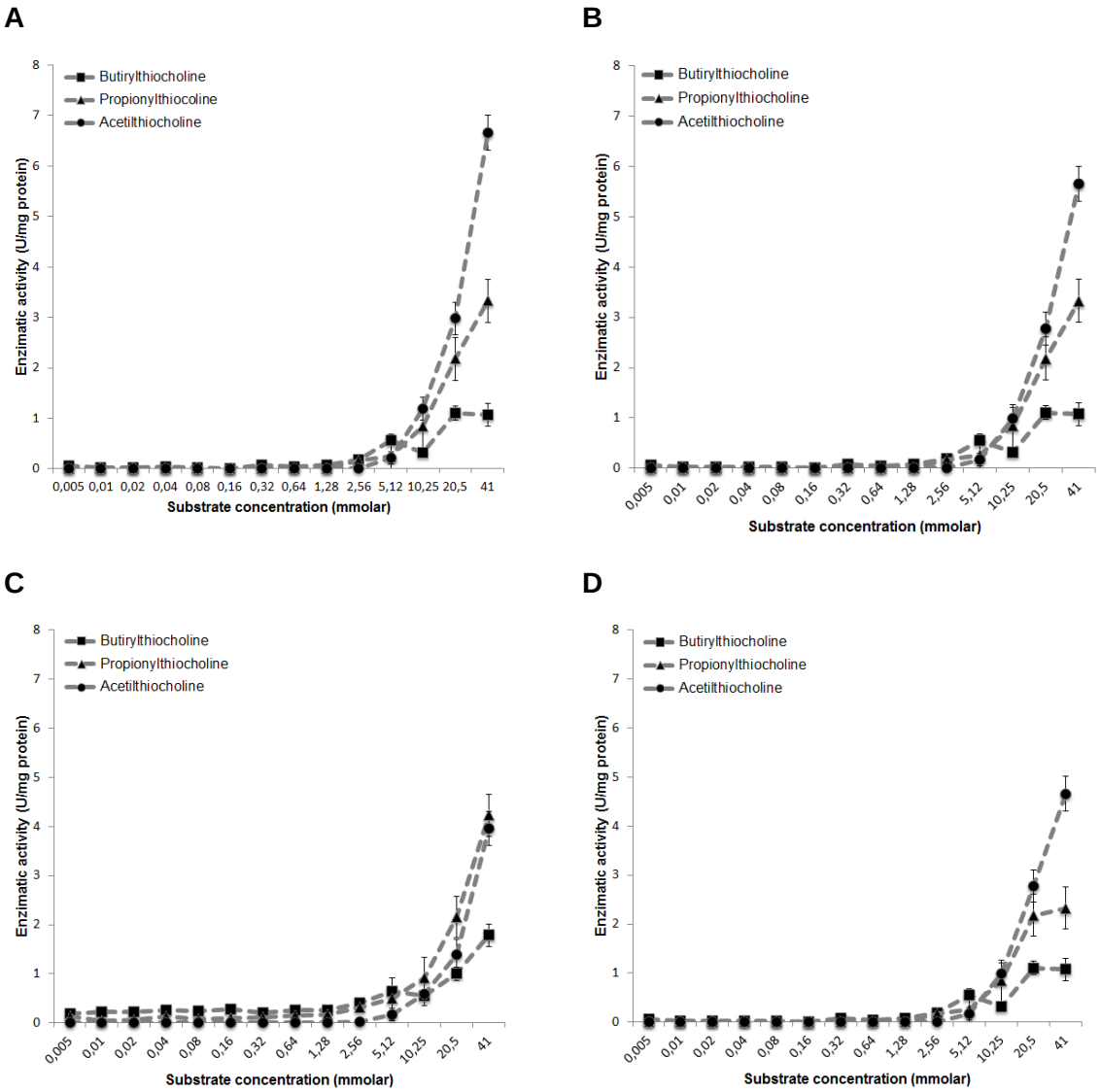

Figure 8 - Cholinesterasic activity determined in the foot tissue of *Corbicula fluminea* as a function of acetylthiocholine, propionylthiocholine, butirylthiocholine concentrations Values are the mean of eight replicates and corresponding standard error bars. The letters A, B, C and D correspond to the different sampling sites, M0, M1, M3 and Lima, respectively.

Table 10 – Percentage of inhibition of enzyme activity determined in foot tissue of *Corbicula fluminea* using eserine sulphate as an inhibitor, with respective standard error (S.E.M.).

Eserine sulphate concentration (µM)	% inhibition of activity with eserine sulphate											
	M0			M1			M3			Lima		
6.25	31.77	±	2.96	19.34	±	1.80	36.08	±	5.45	16.19	±	1.22
12.5	38.22	±	3.91	26.97	±	2.76	46.36	±	3.98	17.25	±	0.94
25	31.21	±	3.06	18.68	±	1.83	11.53	±	2.59	18.67	±	1.83
50	34.44	±	7.92	30.46	±	7.62	11.29	±	2.53	28.40	±	6.64
100	40.01	±	4.69	29.08	±	3.41	36.82	±	3.92	23.60	±	0.96
200	40.28	±	3.45	29.40	±	2.52	43.26	±	7.79	22.91	±	1.87

Table 11 – Percentage of inhibition of enzyme activity determined in foot tissue of *Corbicula fluminea* using BW284C51 as an inhibitor, with respective standard error (S.E.M.).

BW284C52 concentration (µM)	% inhibition of activity with BW284C51											
	M0			M1			M3			Lima		
6.25	0	±	0.29	2.50	±	0.17	12.81	±	1.73	20.20	±	1.34
12.5	11.95	±	1.59	7.29	±	0.59	18.08	±	3.01	18.63	±	0.84
25	8.61	±	0.75	12.06	±	1.21	12.95	±	1.91	22.00	±	1.26
50	9.01	±	0.56	17.11	±	1.96	20.09	±	3.77	17.81	±	0.69
100	27.19	±	5.36	28.67	±	5.66	8.11	±	1.03	18.88	±	0.70
200	11.28	±	0.99	13.39	±	1.22	15.34	±	2.47	21.96	±	1.24
400	1.17	±	0.06	9,48	±	0.85	2.87	±	0.44	29.24	±	0.27

Table 12 - Percentage of inhibition of enzyme activity determined in foot tissue of *Corbicula fluminea* using iso-OMPA as an inhibitor, with respective standard error (S.E.M.). C' corresponds to control with ethanol samples.

iso-OMPA concentration (mM)	% inhibition of activity with iso-OMPA			
	M0	M1	M3	Lima
C'	11.55 ± 0,67	0.62 ± 0.04	10.62 ± 0.60	3.32 ± 0,13
0.5	9.31 ± 0,31	0 ± 0.02	5.98 ± 0.14	0 ± 0.10
1	9.74 ± 0,31	2.90 ± 0.22	10.69 ± 0.57	0 ± 0.05
2	6.61 ± 0,05	0.17 ± 0.01	6.88 ± 0.09	0 ± 0.01
4	13.68 ± 0,64	0 ± 0.14	8.50 ± 0.35	11.47 ± 0.48
8	6.62 ± 0,23	0 ± 0.26	7.34 ± 0.39	0 ± 0.03

The obtained results suggest that ChE with an intermediate form between AChE and PChE is found in both adductor muscle and haemolymph from *C. fluminea*. Gills appear to have some extent of ChE, but results are not conclusive, although they seem to lead to similarities with an intermediate form between vertebrate PChE and AChE. By these results, the esterases present in foot tissue from *C. fluminea* could not be confirmed as ChE. Therefore, the most suitable tissues for ChE assessment in *C. fluminea* are the adductor muscle and haemolymph. Previous studies have characterized the ChE present in the whole body of *C. fluminea* (Mora *et al.*, 1999; Ramos *et al.*, 2012), but never in isolated tissues. The findings of this study can be considered consistent with the previous studies, as the found ChE show an intermediate behavior between AChE and PChE. On the other hand, this study gives insight on which tissue should be chosen to assess ChE activity, as the determined activity of ChE in controls using adductor muscle or haemolymph is almost 10 times higher than using the whole body, considering the values reported by Mora *et al.* (1999) e Ramos *et al.* (2012) for *C. fluminea* whole body.

2.5. Acknowledgements

This research was supported by the European Regional Development Fund (ERDF) through the COMPETE - Operational Competitiveness Programme and national funds through FCT – Foundation for Science and Technology, under the projects *"NISTRACKS – Processes influencing the invasive behaviour of the non-indigenous species Corbicula fluminea (Mollusca: Bivalvia) in estuaries - identification of genetic and environmental key*

factors" (PTDC/AAC-AMB/102121/2008; FCOMP-01-0124-FEDER-008556), and "PEst-C/MAR/LA0015/2011". Joana Rocha had a grant of initiation to investigation (BIC) in the scope of the project NISTRACKS. We would like to thank the other members of the team of the project NISTRACKS, especially Pedro Vilares and Cristiana Oliveira for technical help in clam collection.

2.6. References

Bocquene, G., Galgani, F., Truquet, P. 1990. Characterization and assay conditions for use of AChE activity from several marine species in pollution monitoring. *Marine Environmental Research, 30*: 75-89.

Bocquene, G., Roig, A., & Fournier, D. 1997. Cholinesterases from the common oyster (Crassostrea gigas) - Evidence for the presence of a soluble acetylcholinesterase insensitive to organophosphate and carbamate inhibitors. *FEBS Letters, 407*: 261-266.

Bradford, M. M. 1976. Rapid and sensitive method for quantititation of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry, 72*: 248-254.

Corsi, I.; Bonacci, S.; Santovito, G.; Chiantore, M.; Castagnolo, L.; Focardi, S. 2004. Cholinesterase activities in the Antarctic scallop *Adamussium colbecki*: tissue expression and effect of $ZnCl_2$ exposure. *Marine Environmental Research, 58:* 401-6.

Dimitriadis, K.; Gougoula, C.; Anestis, A.; Pörtner, H.; Michaelidis, B. 2010. Monitoring the biochemical and cellular responses of marine Bivalves during thermal stress by using biomarkers. *Marine Environmental Research, 73:* 70–77.

Doherty, F. G. 1990. The asiatic clam, Corbicula spp, as a biological monitor in freshwater environments. *Environmental Monitoring and Assessment, 15*: 143-181.

Ellman, G. L.; Courtney, K. D.; Andres, V.; Featherstone, R. M. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology, 7*: 88-90.

Garcia, L. M.; Castro, B.; Guilhermino, L. 2000. Characterization of cholinesterase from guppy (*Poecilia reticulata*) muscle and its in vitro inhibition by environmental contaminants. *Biomarkers, 5:* 274-284.

Guilhermino, L.; Lopes, M. C.; Carvalho, A. P.; Soares, AMVM. 1996. Acetylcholinesterase activity in juveniles of *Daphnia magna* Straus. *Bulletin of Environmental Contamination and Toxicology, 57*: 979-985.

Hyne, R. V.; Maher, W. A. 2003. Invertebrate biomarkers: links to toxicosis that predict population decline. *Ecotoxicology and Environmental Safety, 54*: 366-374.

Kozlovskaya, V. I.; Mayer, F. L.; Menzikova, O. V.; Chuyko, G. M. 1993. Cholinesterases of aquatic animals. *Reviews of Environmental and Contamination Toxicology, 132:* 117–142.

Kristoff, G.; Guerrero, N.V.; de D'Angelo, A.M.; Cochón, A.C. 2006. Inhibition of cholinesterase activity by azinphos-methyl in two freshwater invertebrates: *Biomphalaria glabrata* and *Lumbriculus variegatus*. *Toxicology*, 222: 185–194.

Lima, I.; Moreira, S.M.; Soares, A.M.V.M.; Guilhermino, L.. 2007. Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the North-western coast of Portugal. *Chemosphere*, *66*: 1230-1242.

Liu, W.; Zhao, G. Y.; Knowles, C. O. 1994. Substrate specificity and inhibitor sensitivity of cholinesterases in homogenates of western flower thrips. *Pesticide Biochemistry and Physiology, 49*: 121-131.

Mahmoud, N.; Dellali, M.; Bour, M. E.; Aissa, P.; Mahmoudi, E. 2010. The use of *Fulvia fragilis* (Mollusca: Cardiidae) in the biomonitoring of Bizerta lagoon: A mutimarkers approach. *Ecological Indicators*, *10:* 696–702.

Mora, P.; Fournier, D.; Narbonne, J. F. 1999. Cholinesterases from the marine mussels *Mytilus galloprovincialis* Lmk. and *M-edulis* L. and from the freshwater bivalve Corbicula fluminea Muller. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology, 122*: 353-361.

Moreira, S.M.; Coimbra, J.; Guilhermino, L. 2001. Acetylcholinesterase of Mytilus galloprovincialis LmK hemolymph: a suitable environmental biomarker. *Bulletin of Environmental Contamination and Toxicology, 67:* 470-475.

Moreira, S.; Moreira dos Santos, M.; Ribeiro, R.; Guilhermino, L. 2004. The Coral Bulker fuel oil spill on the North coast of Portugal: spatial and temporal biomarker responses in *Mytilus galloprovincialis*. *Ecotoxicology, 13:* 619-630.

Payne, J. F.; Mathieu, A.; Melvin, W.; Fancey, L. L. 1996. Acetylcholinesterase, an old biomarker with a new future? Field trials in association with two urban rivers and a paper mill in Newfoundland. *Marine Pollution Bulletin, 32:* 225-231.

Ramos, A. S.; Goncalves, F.; Antunes, S. C.; Nunes, B. 2012. Cholinesterase characterization in *Corbicula fluminea* and effects of relevant environmental contaminants: A pesticide (chlorfenvinphos) and a detergent (SDS). *Journal of Environmental Science and Health Part B-Pesticides Food Contaminants and Agricultural Wastes, 47*: 512-519.

Sousa, R.; Antunes, C.; Guilhermino, L. 2008. Ecology of the invasive Asian clam *Corbicula fluminea* (Müller, 1774) in aquatic ecosystems: an overview. *Annales de Limnologie - International Journal of Limnology, 44:* 85-94.

Thompson, H. M.; Walker, C. H. 1994. Blood esterares as indicators of exposure to organophosphorus and carbamate insecticides. In: *Non-destructive Biomarkers in Vertebrates*. Fossi, M.C.; Leonzio, C. (Eds.). Lewis Publisher, Chelsea, MI, U.S.A., 37-62.

Tim-Tim, A.L.; Morgado, F.; Moreira, S.; Rangel, R.; Nogueira, A.J.; Soares, A.M.; Guilhermino, L.. 2009. Cholinesterase and glutathione S-transferase activities of three mollusk species from the NW Portuguese coast in relation to the Prestige oil spill. *Chemosphere, 77:* 1465-1475.

Valdez Domingos, F.X.; Azevedo, M.; Silva, M.D.; Randi, M.A.; Freire, C.A.; Silva de Assis, H.C.; Oliveira Ribeiro, C.A. 2007. Multibiomarker assessment of three Brazilian estuaries using oysters as bioindicators. *Environmental Research*, *105*: 350–363.

van der Oost, R.; Beyer, J.; Vermeulen, N. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology*, *13:* 57-149.

Wepener, V.; Van Vuren, J.H.J.; Chatiza, F.P.; Mbizi, Z.; Slabbert, L.; Masola, B. 2005. Active biomonitoring in freshwater environments: early warning signals from biomarkers in assessing biological effects of diffuse sources of pollutants*. Physics and Chemistry of the Earth, Parts A/B/C, 30:* 751-761.

Witter, R. F. 1963. Measurement of blood cholinesterase - A critical account of methods of estimating cholinesterase with reference to their usefulness and limitations under different conditions. *Archives of Environmental Health, 6*: 537-564.

Xu, G.; Bull, D. L. 1994. Acetylcholinesterase from the horn fly (Diptera, Muscidae) Biochemical and molecular properties. *Archives of Insect Biochemistry and Physiology, 27:* 109-121.

Chapter III – Biomonitoring and Seasonal Variability of *Corbicula fluminea* Selected Biomarkers in the Minho River Estuary

Biomonitoring and seasonal variability of *Corbicula fluminea* selected biomarkers in the Minho River estuary

Rocha, J.[1,2] and Guilhermino, L. [1,2]

[1] ICBAS - Institute of Biomedical Sciences of Abel Salazar, University of Porto, Department of Populations Studies, Laboratory of Ecotoxicology, Rua de Jorge Viterbo Ferreira, 228, 4050-313, Portugal
[2]CIIMAR – Interdisciplinary Centre of Marine and Environmental Research, Laboratory of Ecotoxicology and Ecology, University of Porto, Rua dos Bragas, 289, 4050-123 Porto, Portugal

3.1. Abstract

The objective of the present study was to perform a preliminary survey on the variation of selected biomarkers determined in the exotic invasive bivalve *Corbicula fluminea* inhabiting the upper part of the tidal freshwater (TFW) area of the Minho River estuary (NW Iberian Peninsula). Clams were collected in one sampling site over one year (from the winter 2011 to the autumn 2012), and biomarkers indicative of oxidative stress, biotransformation, and alterations in the pathways of energy production were determined. Biomarkers data was integrated through the Integrated Biomarker Response (IBR) index. Differences between seasons were found for lipid peroxidation (LPO), gluthatione-S-transferases (GST), catalase (CAT), isocitrate dehydrogenase (IDH) and for octopine dehydrogenase (ODH) activities. These results once more reinforce that while biomonitoring wild populations one should not overlook the possible natural variability of biomarkers

Keywords: biomarkers, biomonitoring, exotic invasive species, *Corbicula fluminea*

3.2. Introduction

The use of biomonitoring programmes allows the identification and the assessment of anthropogenic pollutants in specific areas along the time (Laffon *et al*., 2006; Zorita *et al*., 2007). In aquatic ecosystems, routine measurements in the water column often include various and different abiotic parameters such as temperature, salinity and dissolved oxygen, nutrients, biological measurements of responses (in individuals, populations or

communities, for example) and chemical analysis to determine the presence of chemical contaminants. Ideally, programmes integrating biological, physical and chemical analysis should be carried out because they will provide the maximum of information regarding the ecological status.

The effect of exogenous substances on ecosystems can be assessed by a battery of biomarkers (Burgeot *et al.*, 1996; Mora *et al*., 1999; Marigómez & Baybay-Villacorta, 2003; Moreira & Guilhermino, 2005; Pfeifer *et al.,* 2005; Rank *et al.,* 2007).

Bivalves are no strangers to assessing biological impacts in aquatic environments, for example, mussels such as *Mytilus galloprovincialis* have been used as bioindicator organisms for Environmental Risk Assessment (ERA) (Porte *et al*., 2001) because of a series of characteristics that make them suitable for use in biomonitoring programmes (Lau & Wong, 2003). These features, among others are shared by *C. fluminea*, and give it an interesting standpoint regarding its use as a freshwater bioindicator species. For oxidative stress and detoxification assessment, the activities of glutathione-S transferase (GST), catalase (CAT) gluthathione peroxidase (GPx) and glutathione reductase (GR) along with lipid peroxidation (LPO) levels were measured. Physiological stress was investigated through esterase activity (EST). Activities of isocitrate dehydrogenase (IDH) and octopine dehydrogenase (ODH) were also assessed as to give insight of energy status.

Biomarkers are useful tools on assessing stress responses, nevertheless, sometimes it is quite hard to conjugate all the different information obtained and give a straightforward answer on the actual health status of the studied organism (Chèvre *et al*. 2003). This lead to the development of a tool that helps combining the various determinations to a one single value: the Integrated Biomarker Response (IBR). This is of much help, as it allows one to describe and compare different areas and populations, regarding to stress levels (Beliaeff & Burgeot 2002; Broeg & Lehtonen 2006; Wang *et al.* 2010). In this study, the results of IBR were used to compare the stress levels in *C. fluminea* in the various seasons. The objectives were (i) to study the seasonal variability of *C. fluminea* EST, GST, CAT, GPx, GR, IDH and ODH enzymes and LPO levels in a reference site of the Minho River estuary and to (ii) provide the basis for their further use in more exhaustive environmental biomonitoring programmes.

3.3. Material and Methods

Animal sampling

Organisms were sampled in the TFW area of Minho (M0 location). The sampling site had the following coordinates:

M0 (near Lapela, Friestas): N42º03'22.51'' W8º32'22.51''

Sample preparation

The sacrifice of animals was made after sampling, in the laboratory. Gills and foot tissues were isolated on ice and removed to respective buffers. Gills were weighed and homogenized in phosphate buffer (0.1 M, pH=7.4) in a proportion of 1:10(w/v), using a Ystral GmbH Dottingen homogenizer. All of the homogenizations were done on ice to prevent degradation. After homogenization, 250µL of homogenate was stored with 4 µL BHT for LPO assessment. The remaining samples were then centrifuged at 10000*g* for 20 minutes at 4ºC, using a Sigma Laboratory Centrifuge, model 3K30. Supernatant were uniformed to a protein content of 4mg/mL and stored at -80ºC, for assessment of CAT, GPx and GR. For GST activity, the protein content was uniformed to 1 mg/mL. Foot tissue was separated in 3 parts: one for EST, in phosphate buffer (0.1 M, pH=7.2); other for IDH, in tris buffer (0.5 M, pH=7.8); and the other for ODH, in tris buffer (0.2 M, pH=7.5). Tissues were homogenized using a Ystral GmbH Dottingen homogenizer. All of the homogenizations were done on ice to prevent degradation. The samples were then centrifuged at 3300*g* for 3 minutes at 4ºC, using a Sigma Laboratory Centrifuge, model 3K30. Supernatant were uniformed to a protein content of 1mg/mL and stored at -80ºC.

Biomarkers determination

Esterases (EST)

ChE activity was determined in triplicate by the Ellman method (Ellman *et al.*, 1961) adapted to microplate (Guilhermino *et al.*, 1996). In short, in each assay well, 0.250 mL of reaction buffer (0.5 mL DTNB (5,5'-Dithiobis-(2-Nitrobenzoic Acid)), 14.1 mL phosphate buffer (0.1M, pH=7.2) and 1.0 mL of acetylthiocholine) was added to 0.05 mL of the corresponding biological sample (or buffer, for controls) and then activity was measured at 412 nm for five minutes, at 25ºC, using a microplate reader (SpectraMax M2e).

Glutathione-S-Transferases (GST)

GST activity is measured by the conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm (Habig *et al.*, 1974) adapted to microplate (Guilhermino *et al.*, 1996). In short, in each assay well, 0.250 mL of reaction buffer (48 mL of phosphate buffer (0.2 M, pH=6.5, 1.521 mL of CDNB (60 mM) in ethanol and 8.78 mL of GSH (10 mM) in ultra-pure water) was added to 0.05 mL of gill supernatant, with protein concentrations of 1 mg/mL. Activity was measured at 340 nm, for 5 minutes and 16 seconds, at 25ºC, using a microplate reader (SpectraMax M2e).

Catalase (CAT)

CAT activity is measured through the decomposition of H_2O_2 to O_2 at 240 nm (Clairborne, 1985). In short, 0.950 mL of phosphate buffer (0.05 M, pH=7.0), 0.500 mL of H_2O_2 and 0.05 mL of gill supernatant, with protein concentrations of 4 mg/mL were added to a cuvette, and activity was measured at 240 nm, for 30 seconds, at 25ºC, using a cuvette reader (SpectraMax M2e).

Glutathione Peroxidase (GPx)

To measure the activity of GPx, it was assessed the decrease in NADPH at 340 nm using H_2O_2 as substrate (Flohé *et al.*, 1973). This enzyme is measure in an indirect way, since it uses the glutathione reductase (GR) to measure the reduction of GSSG to GSH that was previously produced by GPx. The procedure used involves adding 0.8 mL of phosphate buffer (0.05 mM, pH=7.0) with 1 mM EDTA, 1 mM sodium azide and 1 U/mL GR; 0.05 mL of GSH 4 mM; 0.05 mL of NADPH 0.8 mM; 0.01 mL of 0.5 mM H_2O_2 to 0.09 mL of gill supernatant. The reaction is read at 340 nm for 1 min, using a cuvette reader (SpectraMax M2e).

Glutathione Reductase (GR)

Glutathione reductase (GR) activity is the reduction of GSSG to GSH with consumption of NADPH to $NADP^+$. It involves the reaction of 0.9 mL of reaction buffer (phosphate buffer (5mM, pH= 7.0) with nicotinamide adenine dinucleotide phosphate (NADPH), glutathione disulfide (GSSG) and DTPA) with 0.1 mL of gill supernatant, according to Cribb *et al*. (1989).The reaction is read at 340 nm for 1 min, using a cuvette reader (SpectraMax M2e).

Lipid Peroxidation (LPO)

The measurement of LPO is determined by measuring the thiobarbituric acid reactive substances (TBARS) (Ohkawa *et al.*,1979). Briefly, in a 15mL tube, 1 mL of 12%

trichloroacetic acid, 0.8 mL of Tris–HCl (60 mM, pH=7.4) with DTPA 0.1 mM and 1 mL of 0.73% thiobarbituric acid were added to 0.2 mL of homogenate. Then the samples goes to an incubation for about 60 minutes at 100ºC, and the 2mL of this is removed and placed on a 2 mL tube and centrifuged at 12 000 g for 5 minutes. LPO levels are then determined reading the absorbance at 535 nm and expressed in nmol TBARS/mg protein using a cuvette reader (SpectraMax M2e).

Isocitrate Dehydrogenase (IDH)

IDH activity is determined by the measurement of NAPH increase at 340 nm according to Ellis & Goldberg (1971) adapted to microplate (Lima *et al.*, 2007). The procedure consists in adding 0.2 mL of the reaction solution containing 40 mL of Tris buffer, 15 mL of Manganese (II) chloride (2 mM) and 15 mL of DL-isocitric acid (7 mM) in ultrapure water with 0.05 mL of homogenized tissue. The reaction occurs with the addition of 0.05 mL of 0.5 mM NADP and is immediately measured at 340 nm during 3 minutes, at 25ºC, using using a microplate reader (SpectraMax M2e).

Octopine Dehydrogenase (ODH)

Octopine dehydrogenase activity was determined by the measurement of the amount of pyruvate consumed due to NADH oxidation, according to Livingstone *et al*. (1990). In short, in each assay well, 0.200 mL of reaction buffer (15 mL L-Arginine, 40 mL Tris buffer (20 mM, pH= 7.5, with 1 mM EDTA and 1 mM DTT), 15 mL NADH and volume completed to 100mL with u.p. water) was added to 0.05 mL of the corresponding biological sample (or buffer, for controls) and 50 mL of sodium pyruvate. The microplate was agitated for 5 minutes and then activity was measured at 340 nm for 3 minutes, at 25ºC, using a microplate reader (SpectraMax M2e).

Protein content

The quantification of protein was done as described by Bradford (1976) and adapted to microplate (Guilhermino *et al.*, 1996). Bovine y-globulins (Sigma-Aldrich, USA) were used as standard and the readings were done at 600 nm. The dying was prepared with 0.25 mL of Bradford reagent (1 mL of Bradford reagent to 4 mL of ultrapure water) and added to 0.01 mL of sample. The readings were done after 15 min. of agitation and protection from light.

Data analysis

All data analysis were performed using SPSS Statistics 21.0© software package. Test of homogeneity of variances (Levene Statistic) was performed to assess the homogeneity of variances. For each assay, one-way analysis of variance (ANOVA) was performed to check differences between with Tukey test was used to assess differences between seasons.

Stress levels in the various seasons were assessed by an Integrated Biomarker Response (IBR) index, as described by Beliaeff and Burgeot (2002), which studies the relative differences between biomarkers responses.

3.4. Results and Discussion

No significant variation in the activities of the enzymes EST, GPx and GR were found (EST: $F_{3, 78} = 0.272$, $p < 0.05$; GPx: $F_{3, 68} = 0.239$, $p < 0.05$; GR $F_{3, 72} = 1.625$, $p < 0.05$). Significant differences among seasons were found for GST ($F_{3, 75} = 23.025$, $p < 0.05$), CAT ($F_{3, 75} = 9.311$, $p < 0.05$), LPO ($F_{3, 79} = 12.439$, $p < 0.05$), IDH ($F_{3, 78} = 10.458$, $p < 0.05$), and ODH ($F_{3, 71} = 4.165$, $p < 0.05$).

GST has lower activities in Winter and Spring, with higher activities being found for Summer and Autumn (Figure 1). CAT has its' lower activity during Spring, and higher for Winter, Summer and Autumn. LPO acts in the opposite manner of CAT, highest activity is found in Spring, while lower activities are found for Winter, Spring and Autumn. Regarding energetic metabolism, IDH is lower in Winter, with the other seasons having higher similar values, while for ODH, the highest activities are found in Winter and Spring, and lowers in Summer and Autumn.

IDH is responsible for aerobic pathways to obtain energy (Lima *et al*., 2007), while ODH is related with anaerobic ones (Livingstone *et al.,* 1990). The activity of both IDH and ODH is increased in the Spring, possibly due to an increase in the energy needed for growth and reproduction (Korniushin & Glaubrecht, 2003; McMahon & Williams, 1986). Nevertheless, this is the only season where the activities of these two enzymes follow the same trend. In the other seasons, IDH and ODH have opposite trends, suggesting that aerobic pathways of obtaining energy are higher in Summer and Autumn, and that anaerobic ones are being more used in Winter and Spring. IBR results shows a greater stress index for spring and autumn, being LPO levels and IDH activity the major responsible for this value in spring, and CAT for autumn levels (Figure 2). In Summer, biomarkers results also pointed out for a particularly high oxidative damage that could mean a potential increase of exposure to

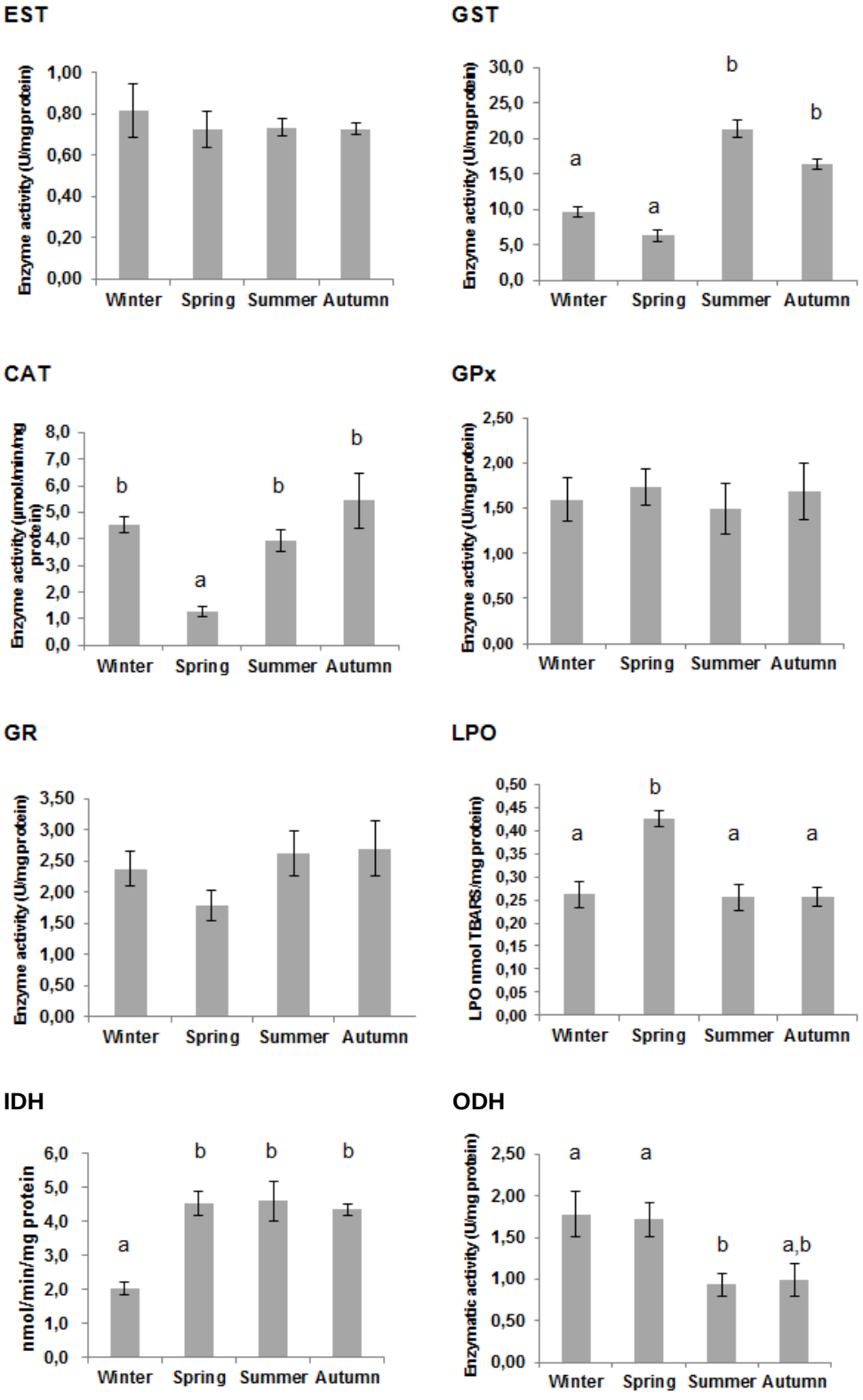

Figure 1- Seasonal variability of Esterases (EST), Glutathione-S-Transferases (GST), Catalase (CAT), Glutathione Peroxidase (GPx), Glutathione Reductase (GR), Lipid Peroxidation (LPO), Isocitrate Dehydrogenase (IDH) and Octopine Dehydrogenase (ODH). The values are the mean of 15 to 20 individuals per season with corresponding S.E.M. bars. Different letters indicate statistical significant differences ($p < 0.05$) after data analysis by one-way Analysis of Variance and the Tukey test.

chemicals during this time of the year, as GST is involved in the metabolism and excretion of exogenous substances. The higher activity of IDH also in spring also contributed for this value of IBR, possibly due to increased energy levels need for both growth and reproduction, as above stated. The effect of variations of GST activity can also be seen in this representation, being this biomarker the major contributor for summer IBR value.

Table 1 – Integrated Biomarker Response (IBR) values for the studied seasons

Season	Winter 2011	Spring 2012	Summer 2012	Autumn 2012
IBR Value	1.176845577	5.584247078	2.31348939	4.133872471

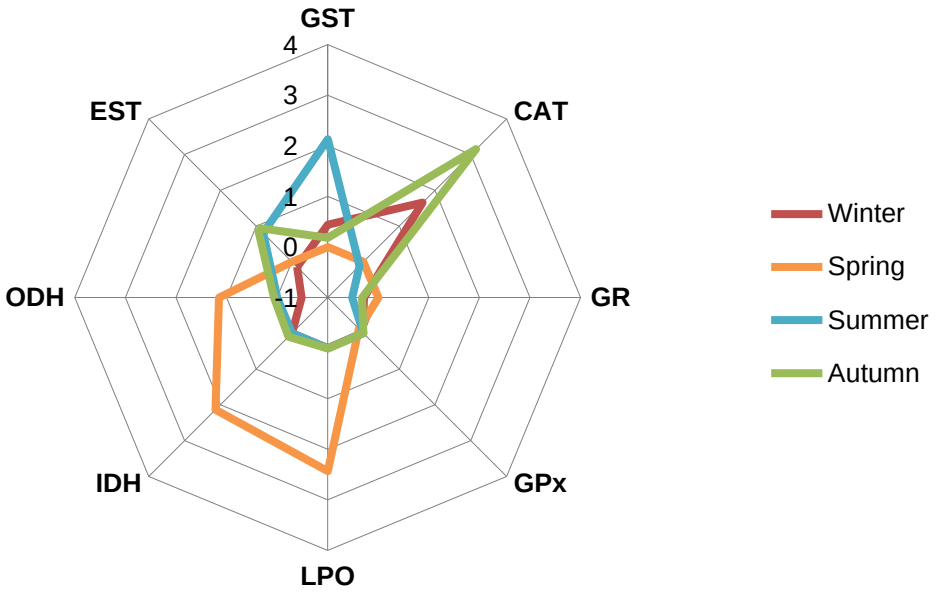

Figure 2 - Integrated Biomarker Response (IBR) station star plot for each season and biomarker from M0 *Corbicula fluminea* samples.

3.5. Acknowledgements

This research was supported by the European Regional Development Fund (ERDF) through the COMPETE - Operational Competitiveness Programme and national funds through FCT – Foundation for Science and Technology, under the projects *"NISTRACKS – Processes influencing the invasive behaviour of the non-indigenous species Corbicula fluminea (Mollusca: Bivalvia) in estuaries - identification of genetic and environmental key factors"* (PTDC/AAC-AMB/102121/2008; FCOMP-01-0124-FEDER-008556), and "PEst-C/MAR/LA0015/2011". Joana Rocha had a grant of initiation to investigation (BIC) in the scope of the project NISTRACKS. We would like to thank the other members of the team of the project NISTRACKS, especially Pedro Vilares and Cristiana Oliveira for technical help in clam collection.

3.6. References

Beliaeff, B.; Burgeot, T. 2002. Integrated biomarker response: a useful tool for ecological risk assessment. *Environmental Toxicology and Chemistry, 21:* 1316–22.

Bradford, M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry*, *72:* 248–59.

Broeg, K.; Lehtonen, K.K. 2006. Indices for the assessment of environmental pollution of the Baltic Sea coasts: integrated assessment of a multi-biomarker approach. *Marine Pollution Bulletin*, *53:* 508–22.

Burgeot, T.; Woll, S.; Galgani, F. 1996. Evaluation of the Micronucleus Test on Mytilusgalloprovincialis for Monitoring Applications along French Coasts. *Marine Pollution Bulletin, 32*: 39-46.

Chèvre, N.; Gagné, F.; Gagnon, P.; Blaise, C. 2003. Application of rough sets analysis to identify polluted aquatic sites based on a battery of biomarkers: a comparison with classical methods. *Chemosphere 51:* 13–23.

Clairborne, A. 1985. Catalase activity. In: *CRC handbook of methods in oxygen radical research*. Greenwald, R.A. (Ed). CRC Press, Boca Raton 283-284.

Cribb, A.E.; Leeder, J.S.; Spielberg, S.P. 1989. 5,5'-dithiobis(2-nitrobenzoic acid). *Analytical Biochemistry*, *183:* 195–196.

Ellis, G.; Goldberg, D.M. 1971. An improved manual and semi-automatic assay for NADP-dependent isocitrate dehydrogenase activity, with a description of some kinetic properties of human liver and serum enzyme. *Clinical Biochemistry, 2:* 175–185.

Ellman, G. L.; Courtney, K. D.; Andres, V.; Featherstone, R. M. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology,* 7: 88-90.

Flohé, L.; Gunzler, W. A.; Schock, H. H. 1973. Gluthathione Peroxidase: A selenoenzyme. *FEBS Letters, 32*: 32-34.

Guilhermino, L.; Lopes, M. C.; Carvalho, A. P.; Soares, AMVM. 1996. Acetylcholinesterase activity in juveniles of *Daphnia magna* Straus. *Bulletin of Environmental Contamination and Toxicology, 57*: 979-985.

Habig, W.H.; Pabst, M.J.; Jacoby, W.B. 1974. Glutathione S-transferases. The first enzimatic step in mercapturic acid formation. *The Journal of Biological Chemistry, 249:* 7130-7139.

Korniushin, A. V.; Glaubrecht, M. 2003. Novel reproductive modes in freshwater clams: brooding and larval morphology in Southeast Asian taxa of *Corbicula* (Mollusca, Bivalvia, Corbiculidae). *Acta Zoologica*, *84:* 293–315.

Laffon, B.; Rábade, T.; Pásaro, E.; Méndez, J. 2006. Monitoring of the impact of acetylcholinesterase activity, a common pollution marker, in Mytilus sp. From the south-western Baltic Sea. *Journal of Experimental Marine Biology and Ecology, 320*: 93-103.

Lau, P.; Wong, H. 2003. Effect of size tissue parts and location on six biochemical markers in the green-lipped mussel, *Perna viridis*, *Marine Pollution Bulletin*, *46:* 1536-1572.

Lima, I.; Moreira, S.M.; Osten, J.R.; Soares, A.M.V.M.; Guilhermino, L. 2007. Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the North-western coast of Portugal. *Chemosphere* 66, 1230–1242.

Livingstone, D.R. 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. *Marine Pollution Bulletin*, *42:* 656–66.

Marigómez, I.; Baybay-Villacorta, L. 2003. Pollutant specific and general lysosomalresponses in digestive cells of mussels exposed to model organic chemicals. *Aquatic Toxicology, 64*: 235 - 257.

McMahon, R.F.; Williams, C.J. 1986. A reassessment of growth rate, life span, life cycles and population dynamics in a natural population and field caged individuals of *Corbicula fluminea* (Müller) (Bivalvia: Corbiculacea). *American Malacological Bulletin*, *2:* 151–166.

Mora, P.; Fournier, D.; Narbonne, J. F. 1999. Cholinesterases from the marine mussels *Mytilus galloprovincialis* Lmk. and *M-edulis* L. and from the freshwater bivalve Corbicula fluminea Muller. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology, 122*: 353-361.

Moreira, S.M.; Guilhermino, L. 2005. The use of Mytilus galloprovinciallis acetylcholinesterase and gluthatione S-transferase activities as biomarkers of environmental contamination along the northwest Portuguese coast. *Environmental Monitoring and Assessment, 105*: 309-325.

Ohkawa, H.; Ohishi, N.; Yagi, K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, *95:* 351-358.

Pfeifer, S., Schiedek, D.; Dippner, J.W. 2005. Effect of temperature and salinity on acetylcholinesterase activity, a common pollution biomarker, in Mytilus sp. from the south-western Baltic Sea. *Journal of Experimental Marine Biology and Ecology 320:* 93–103.

Porte, C.; Biosca, M.; Solé, M.; Albaigés, J. 2001. The integrated use of chemical analysis, cytochrome P450 and stress proteins in mussels to assess pollution along the Galician coast (NW Spain). *Environmental Pollution*, *112:* 261-268.

Rank, J.; Lehtonen, K.; Strand, J.; Martha Laursen, M. 2007. DNA damage,acetylcholinesterase activity and lysosomal stability in native and transplanted mussels(*Mytilus edulis*) in areas close to coastal chemical dumping sites in Denmark. *Aquatic Toxicology, 84*: 50-61.

Wang, C.; Lu, G.H.; Song, W.T.; Xu, S.; Wang, P.F. 2010. Integrated biomarker response index for the assessment of environmental stress of the Yangtze River (Nanjing section). *Fish Physiology and Biochemistry*, *36:* 1069–78.

Zorita, I.; Ortiz-Zarragoitia, M.; Apraiz, I.; Cancio, I.; Orbea, A.; Soto, M.; Marigómez, I.; Cajaraville, M.P. 2007. Assessment of biological effects of environmental pollution along the NW Mediterranean Sea using mussels as sentinel organisms. *Environmental pollution*, *148:* 236–50.

Chapter IV – Conclusions and Future Perspectives

The work present in this thesis aimed to (i) characterize the ChE enzymes present in distinct tissues (adductor muscle, foot, gills and haemolymph) of *Corbicula fluminea* from the estuaries of Minho and Lima Rivers; and to (ii) study the seasonal variability of *Corbicula fluminea* EST, GST, CAT, GPx, GR, IDH and ODH enzymes and LPO levels in a reference site of the Minho River estuary providing the basis for their further use in more exhaustive environmental biomonitoring programmes.

The results of this study indicate that, in adductor muscle, haemolymph and gills, there is present a form of ChE that has characteristics of both AChE and PChE. Foot tissue presented an enzyme activity that could be classified as of EST.

In biomonitoring studies with this species, specific tissues from *Corbicula fluminea* should be used to measure ChE or EST activities instead of using the whole body. Adductor muscle or haemolymph seem to be the most suitable tissues for assessing ChE activity, while foot tissue should be used for for EST activity.

While assessing *C. fluminea* health status, seasonal variability should be considered as it can bias the interpretation of results. Also, the monitored site in Minho river can be used as a reference in future studies, as specimens show relative low values of environmental stress.

Further studies should be made for providing ecotoxicological tools for use in Forensic Medicin. For example, in establishing the circumstances of dead through the identification of particular toxins and/or organisms present in the local of the fatality, using animal models in ecotoxicological assays to diagnose modes of toxic action, symptoms and mortality due to the exposure to the agent(s) suspected of having cause the human fatality, among other possibilities.